

MODULATION OF FOETAL PANCREAS

IMMUNOGENICITY

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STATEMENT

Transplantation studies of isolated adult mouse islets presented in Chapter 4 and Chapter 6 were performed by Dr K.M. Bowen. Dr J.A. Donohoe was responsible for assessing the function of mouse thyroid grafts described in Chapter 6. With these exceptions, the experiments presented in this thesis represent my own original work.

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ABSTRACT

The immunogenicity of foetal mouse pancreas can be substantially reduced by treating the donor tissue *in vitro* before allotransplantation. Two procedures are shown to be effective in modulating foetal pancreas immunogenicity:

- (i) organ culture for 20 days in a 95% O₂, 5% CO₂ gas phase; and
- (ii) the preparation of foetal proislets.

Allografts of 20 day-cultured foetal pancreas tissue, transplanted across a major histocompatibility barrier, demonstrate the capacity to reverse Streptozotocin-induced diabetes in a proportion of recipient mice.

A comparative study of the immunogenicity of pancreatic islet tissue derived from different sources provides evidence for tissue-specific properties. The organ culture technique is much more effective in conditioning isolated adult islets for allotransplantation than in the case of the foetal pancreas. In contrast to the prolonged duration of culture necessary to show evidence of acceptance of foetal pancreas allografts, only 7 days of culture in the same gas phase are required to achieve indefinite survival of allografts of adult islets transplanted across the same major histocompatibility barrier. Evidence that foetal pancreas explants are contaminated with primitive lymphoid tissue forms the basis for our proposal that tissue-specific differences in immunogenicity are related to the degree of contamination of graft tissue by immunostimulatory passenger leukocytes. Thus we suggest that the effectiveness of *in vitro* procedures to

modulate the immunogenicity of foetal pancreas tissue can be attributed to the extent to which the associated lymphoid component is removed before allotransplantation and hence to the degree of elimination of passenger leukocytes capable of activating a graft rejection response.

Tissue-specific differences in immunogenicity are shown to be pronounced following transplantation across a minor histocompatibility barrier. In contrast to the high immunogenicity of untreated foetal pancreas and skin, isolated foetal proislets, isolated adult islets and thyroid are weakly immunogenic. Both organ culture in a high oxygen gas phase and the preparation of foetal proislets are effective in reducing the immunogenicity of foetal pancreas allografts transplanted across a minor histocompatibility barrier. These findings support our proposal that tissue-specific effects in immunogenicity can be attributed to the quantity of passenger leukocytes or fixed lymphoreticular elements carried in the transplant tissue. It is suggested that, in the case of only minor histocompatibility differences, passenger leukocytes render tissue transplants susceptible to non-specific damaging reactions.

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FOETAL MOUSE PROTEIN IS

PREFACE

This thesis is entitled "Modulation of foetal pancreas immunogenicity" and is divided into seven chapters. The introductory chapter (Chapter 1) reviews the literature on transplantation immunobiology mainly up until 1979, at which point this project was commenced. Particular attention is paid to the "traditional" concept that transplantation antigen represents the barrier to allotransplantation. It is our hypothesis that foreign antigen is not the major barrier to successful allotransplantation and we examine the supportive evidence provided by *in vivo* and *in vitro* models of alloreactivity. These findings are reinforced by transplantation studies which demonstrate that allograft immunogenicity can be substantially reduced by treating the donor tissue *in vitro* prior to transplantation.

In this thesis we set out to investigate the generality of this *in vitro* approach to the modulation of tissue immunogenicity, adopting, in particular, the organ culture technique. We have chosen the foetal mouse pancreas as our principal tissue for investigation and our analysis embraces comparative studies of the immunogenicity of foetal pancreas, isolated adult islets, proislets, thyroid and skin. Chapter 3 examines the optimal culture conditions required to maintain foetal mouse pancreas *in vitro*; Chapter 4 compares the capacity of organ culture to reduce the immunogenicity of foetal pancreas and isolated adult islets allografted across the same major histocompatibility barrier; Chapter 5 examines the immunogenicity of isolated foetal mouse proislets and Chapter 6

details a comparison of the immunogenicity of foetal pancreas, proislets, isolated adult islets, thyroid and skin transplanted across the same minor histocompatibility barrier. Chapter 2 describes the materials and methods employed in this project and Chapter 7 relates the experimental findings of the project to the current literature and consequently should be considered as an extension of the overview presented in Chapter 1.

ABBREVIATIONS and SYMBOLS

ALS	Antilymphocyte serum
ATXBM	Adult thymectomized, lethally irradiated and bone marrow reconstituted
α	alpha
β	beta
BSA	Bovine serum albumin
ConA	Concanavalin A
c.p.m.	counts per minute
DNase	Deoxyribonuclease
DTH	Delayed type hypersensitivity
EMEM	Eagle's Minimal Essential Medium
g	acceleration due to gravity
gm	gram
GVH	Graft versus host
HBSS	Hanks Balanced Salt Solution
^{125}I	^{125}I Iodine
Ia	I region associated
Ig	Immunoglobulin
Ir	Immune response
M	Molar
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
mmoles	millimoles
mmol/l	millimoles per litre
O.D.	Optical density
PBS	Phosphate Buffered Saline
PC	Peritoneal cells
SD	Standard deviation
TDL	Thoracic duct lymphocytes
T.TDL	T cell-enriched thoracic duct lymphocytes
U	Units
UV	Ultraviolet
μ	micron
μCi	microcurie
v/w	unit volume per unit weight

CHAPTER 1

THE IMMUNOBIOLOGY OF TISSUE TRANSPLANTATION

1.1 EARLY CONCEPTS OF GRAFT REJECTION

Pioneer studies of tissue transplantation in the early 1900s indicated that the capacity of mice to accept or reject transplantable murine tumours was inherited (Tyzzer, 1909). Speculations about the mechanism of this inheritance were confused by the use of genetically undefined populations. Further progress in this area awaited the development of inbred animal lines from repeated brother-to-sister matings. Knowledge of the genetic background of transplant donors and recipients permitted a systematic study of tissue transplantability. Using inbred mouse colonies, Little (1921-1922) established that the susceptibility of hybrid and backcross generations to a range of transplantable murine tumours was inherited according to Mendelian laws and was attributable to the independent segregation of multiple genes which later became known as "histocompatibility" genes (Snell, 1948). This demonstration marked the inception of the study of transplantation genetics (Little, 1924).

The process of graft rejection was seen by Loeb (1918a) to reflect a biochemical difference between the tissues of the donor and host. From a conceptual viewpoint, the chemical substance of an individual which distinguished it from another individual was called the "individuality differential" (Loeb, 1918a). Moreover, Loeb saw this differential as the product of the genes which controlled tissue compatibility (Loeb, 1930). According to his concept, the individuality differential of an allograft was converted to a toxin following transplantation, possibly via an interaction with the "body fluids" of the foreign host. This toxin activated

the host's cellular response, a process which culminated in graft destruction (Loeb, 1930). In other words, graft rejection resulted from a two-way interaction between the graft and host tissues.

Loeb's radical ideas of tissue transplantation were opposed by other theories which advocated immunity and "athrepsia" as possible mechanisms of graft rejection. The latter theory proposed that graft rejection resulted from the failure of transplanted tissues to compete with host tissues for essential nutrients (Loeb, 1930). However, this concept lacked supportive evidence. Support for the other alternative theory was provided by the demonstration of active immunity to tumour allografts (Tyzzer, 1916). Nevertheless, attempts by others to demonstrate transplantation immunity to normal tissues were inconsistent and Loeb himself failed to observe an accelerated rejection reaction to secondary thyroid allografts in guinea pigs (Loeb, 1918b). This finding favoured the theory of the individuality differential as the trigger for graft rejection (Loeb, 1918b, 1930). It should be pointed out here that much of the early confusion concerning the role of active immunity in the rejection of normal tissue transplants can be attributed to the use of multiple donors belonging to outbred or only partially inbred animal strains (Loeb, 1918b; Section 1.2).

The individuality differential theory of graft rejection emphasized an active contribution of the graft itself. Consequently Loeb questioned "whether it might not be possible to alter the tissues before transplantation in such a way that they might still live and perhaps even regenerate, without however producing those substances which

call forth the lymphocytic reaction" (Loeb, 1930). This notion was supported by the better survival of cartilage and perichondrium tissues which had been exposed to moderately high temperatures prior to allotransplantation (Loeb, 1930).

Using a different approach, Erdmann obtained prolonged survival of allografts of embryonic bird tissues and amphibian skin xenografts by cultivating the donor tissue *in vitro* in the "body fluids" of the prospective host, prior to transplantation (see Loeb, 1930). The improved graft survival was attributed to a gradual adaptation of the donor tissue to the foreign host environment, simulated *in vitro*. Moreover, these findings suggested that the individuality differential was in some way inactivated during the conditioning process. Long-term survival of canine thyroid grafts was similarly obtained following *in vitro* culture in a cocktail of the serum and plasma of the future host (Stone *et al.*, 1934). In addition, there were reports of clinical recovery from hypoparathyroidism following transplantation of human parathyroid tissue which had been pretreated by organ culture in the "body fluids" of the recipient (Stone *et al.*, 1934; Gaillard, 1948). However, these experiments were not genetically controlled and the lack of direct evidence of graft survival in clinical studies failed to exclude the possibility of spontaneous recovery. Consequently this culture technique was treated with considerable scepticism and was not pursued.

From an historical standpoint, it is noteworthy that even as early as the 1930s modification of the donor tissue prior to transplantation was seen as an approach to preventing graft rejection.

1.2 GRAFT REJECTION AS AN IMMUNE PROCESS

Studies of tumour transplantation by Gorer provided the first conclusive evidence for an immune reaction to foreign tissue. The presence of donor-specific antibodies in mice carrying rejected allogeneic tumour grafts suggested that foreign antigens carried on the donor tissue played a role in activating the host's rejection response (Gorer, 1937). However, it was not until Medawar's classical studies of skin graft rejection in rabbits that the rejection process was shown unequivocally to represent an actively acquired immune response. This conclusion was based on the accelerated or "second-set" rejection of a secondary skin allograft transplanted to hosts carrying a primary allograft from the same donor. The recall was specific for the primary donor type and the rejection response was systemic (Medawar, 1944). Together, these properties fulfilled the definition of *active immunity*.

1.2.1 Evidence for a relationship between antigen recognition and graft rejection.

In subsequent studies, Medawar demonstrated that second-set rejection of skin allografts was elicited equally well by prior immunization of the hosts with blood leukocytes of the same donor specificity. On the basis of this finding Medawar proposed that the leukocytes and skin expressed common antigens (Medawar, 1946). Implicit in this explanation was the notion that recognition of foreign antigen activated the graft rejection response. This idea later formed the basis of the "traditional" concept of graft rejection (Section 1.2.3).

Addressing the nature of immune reactivity in a much broader context, Burnet and Fenner (1949) developed a theoretical model based on the principle that contact with foreign antigen led to expression of immune responsiveness. To account for the discrimination between self and non-self (i.e. foreign) determinants, they postulated that non-reactivity to self-components was learnt early during ontogeny. Thus, it was predicted that exposure of foreign antigen to an individual during embryonic life should prevent the development of immune reactivity and hence induce a state of antigen-specific "tolerance" in later life. This notion subsequently revolutionized the direction of immunological research. However, initial attempts to verify the postulate were unsuccessful. Using the chick embryo model and antibody production as a test for immune responsiveness, Burnet *et al.* (1950) failed to demonstrate tolerance to a range of antigens including influenza virus, bacteriophage C16 and human red blood cells.

At the same time, Medawar and his colleagues were seeking ways to overcome the immunological problem of allograft rejection. Consequently they chose to test the principle of tolerance induction. Foetal mice of a highly inbred strain were immunized *in utero* with a composite cell suspension of allogeneic spleen, kidney and testis tissues. As adults, the mice were challenged with a skin graft of the same donor specificity. The long-term survival of a proportion of grafts provided convincing evidence for the active induction of immunological tolerance. Additional studies clearly showed that the tolerance was specific for the

cell donor strain and the phenomenon was confirmed in other animal models including the chick embryo, newborn mouse and chick (Billingham *et al.*, 1953, 1956a).

The discrepancy between these studies was later traced to the persistence of the test antigen(s) following host inoculation (Smith and Bridges, 1958; Mitchison, 1959). In any case, the demonstration that graft rejection could be prevented by inhibiting antigen recognition provided conclusive evidence that the rejection response represented an immune phenomenon.

1.2.2 The cellular nature of allograft rejection

Further efforts to analyse the mechanism of graft rejection focussed on the capacity of various cell and cell-free fractions to passively transfer transplantation immunity to naive recipients. Using this approach, Mitchison (1953, 1954) found that by prior treatment of naive hosts with a suspension of regional lymph nodes taken from mice sensitized by a tumour allograft, tumour allografts of the same specificity were rejected in second-set fashion. However, immune serum, peritoneal cells and non-regional lymph node cells from the same sensitized donors were ineffective in transferring immunity (Mitchison, 1953, 1954). Billingham *et al.* (1954) confirmed this phenomenon by showing that immunity to murine skin allografts could be similarly transferred using immune lymph node cells or spleen cells but not serum, whole blood or blood leukocytes.

Failure to demonstrate an effect of immune serum in this system argued against a direct contribution of antibody to acute graft destruction. Thus, graft rejection was recognized

as a cell-mediated event. Cytological analyses of lymph nodes draining the site of tissue allografts subsequently identified a specific cellular response characterized by proliferation and blastogenesis (Scothorne, 1957). These findings provided strong evidence for the notion that the regional lymph nodes represented the primary site of immune reactivity. This property was consistent with their superior capacity to passively transfer transplantation immunity.

Further support for the cellular basis of graft rejection was shown when adult mice, which had been rendered tolerant during foetal life, demonstrated a restored capacity to reject tolerized skin allografts after receiving an inoculum of isogeneic normal or immune lymph node cells (Billingham *et al.*, 1954). Gowans *et al.* (1962) used a similar experimental system to show that F_1 skin allografts carried by tolerant rats could be rejected following the transfer of isogeneic thoracic duct lymphocytes. However, the contribution of lymphocytes to the graft rejection process was somewhat confused by the popular concept that all lymphocytes represented the precursors of antibody-forming cells (Gowans *et al.*, 1962). This notion was clearly inconsistent with the failure of immune serum to transfer transplantation immunity. Nevertheless, the data reinforced the cellular nature of graft rejection. It should be pointed out here that the unsuccessful attempt by Billingham and co-workers (1954) to transfer transplantation immunity using blood leukocytes was probably due to the low level of sensitized lymphocytes circulating in the blood early after sensitization.

1.2.3 The "traditional" concept of graft rejection

On the basis of the experimental evidence already discussed (Section 1.2), an overall picture of the events leading to graft rejection was promptly formulated. According to Medawar's interpretation, graft antigens were passively transported to the regional lymph nodes where they activated the rejection response (Medawar, 1956-1957). In other words, Medawar saw that antigen recognition was sufficient requirement for lymphocyte activation (Medawar, 1963). In addition, he proposed that all nucleated cells of an individual carried the same transplantation antigens (Medawar, 1956-1957). These ideas offered a simple explanation for two important observations: firstly, the capacity of blood leukocytes to immunize against skin allografts of the same donor specificity and secondly, the induction of tolerance to skin allografts by early exposure to donor-specific antigens on other tissues, including spleen. Further support for his concept was provided by the demonstration that crude cell-free extracts of allogeneic spleen, thymus and lymph node tissues could provoke the accelerated rejection of skin allografts of the same donor type (Billingham *et al.*, 1958).

Even so, the phenomenon of immunological enhancement presented an obvious paradox to the hypothesized role for antigen in graft rejection. In this system, prior immunization with homogenates or extracts of fresh, frozen or lyophilized tissues substantially prolonged the survival of tumour allografts of identical donor specificity (Snell, 1952; Day *et al.*, 1954). Similarly, Billingham *et al.* (1956b)

reported enhanced survival of skin allografts in mice by pretreatment with lyophilized preparations of kidney, spleen and liver tissues. The demonstration that graft enhancement could be achieved by the passive transfer of the globulin fractions of donor-specific antiserum established an antibody-mediated mechanism (Kaliss and Kandutsch, 1956) and hence a role for antigen in the induction of active enhancement. Graft rejection and enhancement, the "Yin" and "Yang" of transplantation biology, therefore appeared to share antigen as the active agent.

Nevertheless, Medawar's postulate that foreign antigens on graft parenchymal cells represented the barrier to allotransplantation was widely accepted and in more recent years acquired the title of the "traditional" concept of graft rejection. Moreover, an attractive explanation for the immunological and genetic contributions (Section 1.1) to graft rejection was the idea that tissue antigens were membrane-bound products of the histocompatibility genes which controlled tissue transplantability (Snell, 1957a). On these grounds, the technique of organ culture, mentioned earlier (Section 1.1), was strongly criticized as an "infirm" attempt "to change the antigenic constitution of a graft" (Medawar, 1957) and hence to modulate gene expression. In compliance with Medawar's doctrine, the principle of pretreating donor tissue prior to grafting was abandoned and attention turned to ways of modifying host immune responsiveness. Two basic methods were pursued: host immunosuppression using corticosteroids, irradiation or antilymphocyte serum (Medawar, 1957; Woodruff and Anderson, 1964) and tissue matching, a procedure developed

specifically for clinical transplantation (Goldsmith, 1965).

Extensive immunogenetic studies have shown now that transplantation antigens are determined by a tight assembly of genes called the major histocompatibility complex or MHC.

1.3 THE MAJOR HISTOCOMPATIBILITY COMPLEX

The MHC of the mouse, commonly referred to as the H-2 complex, has been reviewed recently by Klein (Klein, 1979; Klein *et al.*, 1981). A summary of this information forms the basis of this section.

The H-2 complex is located on chromosome 17 and is divided into three main regions designated *K*, *I* and *D*. The *K* and *D* regions code for membrane-bound glycoproteins with a molecular weight of 45,000 daltons. The close homology in the primary structure of these products persuaded Klein (1979) to classify them together as class I molecules. Each molecule consists of a single polypeptide chain and is noncovalently associated with a small polypeptide called β_2 -microglobulin. This smaller molecule has a molecular weight of 12,000 daltons and is encoded by a locus located on chromosome 2. The composite products are referred to as H-2 antigens. The *I* region codes for cell surface glycoproteins composed of two noncovalently linked polypeptide chains called α and β . The α and β molecules have molecular weights of approximately 32,000 and 28,000 daltons respectively. The *I* region products are called class II molecules and they form the *I* region-associated or *Ia* antigens. Unlike class I antigens which appear to be represented on all body tissues, class II antigens display a restricted tissue distribution. The *Ia*

antigens are detected principally on lymphoid tissues, due to their expression on macrophages as well as on both T and B lymphocytes (Hämmerling, 1976).

The H-2 complex is extremely polymorphic and both the class I and class II loci display a large range of alleles. The allelic composition of the H-2 complex is referred to as the haplotype and is designated by an alphabetical superscript, e.g. H-2^d. The molecular products of histocompatibility loci located outside the MHC are less well defined and are referred to as minor or non-H-2 histocompatibility antigens (Klein, 1975a).

Similar MHC systems have been described for a number of species including man, rat, pig, monkey, chicken and dog (Klein, 1975b). Amino acid sequencing studies have indicated that class I molecules show strong homology amongst different species (Klein, 1979).

Although the H-2 loci were originally defined as genes controlling tissue transplantation, *in vitro* studies have indicated that the basic function of the MHC is to control immune responsiveness in a much broader capacity. This regulation is achieved at the level of thymus-derived or T lymphocytes (Klein *et al.*, 1981). The H-2 complex restricts the specificity of T cells activated against virus-infected targets, controls the provision of T cell "help" required for the differentiation of B lymphocytes into antibody-producing cells, and harbours immune response (*Ir*) genes some of which control helper T cell reactivity and hence the level of antibody production to a range of antigens. The MHC also determines the reactivity of mixed lymphocyte cultures (MLCs).

In a one-way MLC where responder T cells are mixed with H-2 incompatible lymphoid cells, pretreated either by gamma-irradiation or Mitomycin C to inhibit cell proliferation, the responding T cell population is stimulated to proliferate and express cytotoxic activity specific for targets bearing the H-2 type of the stimulating cell population. In particular, the H-2 complex controls the activation of different subclasses of T lymphocytes which are distinguished by the expression of T cell-specific Lyt antigens: Lyt-1, Lyt-2 and Lyt-3 (Cantor and Boyse, 1975). T cells activated against class I antigens carry the $\text{Lyt-1}^+2^+3^+$ phenotype; those responsive to class II structures express only the Lyt-1^+ antigen, i.e., $\text{Lyt-1}^+2^-3^-$ (Cantor and Boyse, 1975; Ledbetter *et al.*, 1980).

This array of H-2 controlled traits strongly suggests a role for T cells in graft rejection. The experimental evidence for this notion will be examined in detail, in the following section.

1.4 MECHANISMS OF GRAFT REJECTION

Experimental evidence presented in an earlier section (Section 1.2) supported the claim that graft rejection was a cell-mediated immune response. Moreover, the capacity for transferred lymphocytes to abrogate an experimentally-induced state of tolerance to tissue allografts argued a central role for lymphocytes in the rejection reaction. In this section, the evidence favouring the cellular basis of graft rejection is expanded to reveal the process as a complex network of immune mechanisms.

1.4.1 Evidence supporting a role for T cells in graft rejection

Studies performed on neonatally thymectomized mice demonstrated prolonged survival of skin allografts and xenografts, coupled with severe depletion of the lymphocyte sub-population of lymph nodes and spleen (Miller, 1961; 1962). Similar findings were reported for the rat, hamster and chicken (Miller and Osoba, 1969). Reconstitution of thymectomized neonatal mice with histocompatible foetal or neonatal thymus transplants essentially restored the capacity to reject allogeneic skin grafts (Miller, 1961; 1962). Taken together, these reports suggested that graft rejection was a thymus-dependent operation. The failure of genetically athymic mice to reject foreign skin grafts (Wortis, 1971; Manning *et al.*, 1973) together with the restoration of allograft reactivity following transplantation of normal thymus (Wortis *et al.*, 1971) provided further support for this notion. The requirement for thymus function, in conjunction with the established capacity of lymphocytes to provoke graft rejection (Section 1.2.2), provided a fairly strong case for the involvement of thymus-derived T lymphocytes in the rejection process.

The adoptive cell transfer system has been used to examine this hypothesis. In these studies, hosts were rendered immunologically incompetent either by neonatal thymectomy or sub-lethal irradiation. Using the former procedure, Sprent and Miller (1972a) inoculated P_1 mice bearing intact P_2 and P_3 skin allografts, with thoracic duct lymphocytes (TDL) derived from a population of P_1 thymocytes

(T cell precursors) which had been sensitized *in vivo* to P_2 alloantigens, i.e. "T.TDL". The specific and accelerated rejection of the P_2 allograft was attributed to the contribution of activated T cells to the immune response. However, while these findings strongly suggest a major role for T cells in mediating the rejection response, the data are not conclusive. This criticism is based on the cellular composition of the T.TDL inoculum and the experimental design. Analysis of the T.TDL population with specific antisera and complement revealed that up to 96% of the cells expressed the thymocyte-lineage-specific θ antigen, but did not carry kappa, an immunoglobulin marker for B lymphocytes (Sprent and Miller, 1972b). Therefore, the T.TDL represented a highly enriched but impure population of T cells. In addition, only 60% of the T.TDL were blast cells (Sprent and Miller, 1972b). Thus, a substantial proportion of the inoculum were resting T cells. The possible contribution of non-T cells and resting T cells to the rejection mechanism therefore cannot be excluded. A solution to the former problem would have been to treat the T.TDL population, in a parallel experiment, with anti- θ serum and complement prior to transfer. The continued survival of the relevant skin graft would then have verified that the rejection mechanism was a T cell-dependent phenomenon. However the low cytotoxic titres of the alloantisera prepared at that time may have prevented this approach. Certainly the monoclonal anti- θ sera available today would overcome this technical difficulty. The selective removal of resting T cells from the inoculum would not have been possible at the time of Sprent and Miller's study; using the modern facility of the Fluorescence-Activated Cell Sorter (FACS),

this separation could probably be achieved today.

Hall and colleagues (1978a) transferred various cell populations to sub-lethally irradiated rats carrying functioning heart allografts. They demonstrated that the capacity to restore the graft rejection response was a function of Ig-negative, recirculating, long-lived lymphocytes. Immunofluorescence staining techniques showed that 95% of the recirculating lymphocytes were T cells. The tempo of graft rejection following reconstitution with unfractionated cells (lymph node cells or thoracic duct lymphocytes) was similar to that observed with Ig-negative cells. An important finding was that the absence of a detectable level of antibody in the serum of rats treated with Ig-negative lymphocytes indicated that antibody did not contribute to heart allograft rejection. On the basis of these findings, the authors concluded that T cells were responsible for mediating graft rejection. Once again, however, the small degree of contamination of these inocula with non-T cells renders the evidence for this role suggestive. Furthermore, a different mechanism for second-set rejection was indicated when the memory cells were shown to belong to a distinctly separate pool of Ig-negative, long-lived cells which did not recirculate (Hall *et al.*, 1978b).

More recent reports have presented convincing evidence that T cells mediate the process of graft rejection. These findings are outlined in the final chapter of this thesis (Chapter 7).

1.4.2 The inflammatory cell response

The recovery of cell populations infiltrating rejecting allografts, by enzymatic digestion or mechanical procedures, has revealed that the inflammatory response is heterogeneous and dynamic. Cell fractionation procedures and immunofluorescence techniques have shown that in addition to T cells, a variety of other mononuclear leukocytes participate; these include non-T lymphocytes, B lymphocytes, monocytes, macrophages and null cells. The relative contribution of each cell type to the cellular infiltrate is influenced by a number of parameters: the nature of the tissue allografted, the post-transplant time at which the infiltrate is analysed and, to a lesser extent, the method of cell recovery (for review, see Carpenter, 1981). The development of the sponge matrix allograft model for rodents markedly improved the quantitative recovery of viable cells by overcoming the need for proteolytic digestion. In turn, this system facilitated assessment of the possible contribution of different classes of infiltrating cells to graft destruction (Roberts and Häyry, 1977).

In order to identify potential rejection mechanisms, the various infiltrating cell sub-populations have been tested for the capacity to lyse ⁵¹Chromium-labelled donor-specific targets *in vitro*. In the case of rat heart allografts and murine sponge matrix allografts, treatment of the infiltrate with T cell-specific antiserum plus complement, demonstrated that T lymphocytes were the predominant cytotoxic cell population during the early post-transplant period (Strom *et al.*, 1977; Wiktorowicz *et al.*, 1978; Ascher *et al.*, 1979).

However, at later stages of sponge matrix allograft rejection, the majority of lymphocyte-mediated cytotoxicity was attributable to non-T lymphocytes (Roberts and Häyry, 1977). Enrichment for Fc-receptor-bearing cells using rosetting techniques demonstrated that monocytes and macrophages were also active in target cell lysis (Roberts and Häyry, 1977). In addition, the rat heart allograft model provided evidence for an Fc-receptor-positive cell population, distinct from macrophages, which lysed target cells coated with anti-donor IgG (Strom *et al.*, 1977). Thus, antibody-dependent cell-mediated cytotoxicity was shown to represent another potential mechanism of graft rejection.

These studies have demonstrated that in addition to T cells, several different infiltrating cell types have the potential to express effector cell function. Of interest is the report by von Willebrand and Häyry (1978) that the parenchymal cells of human kidney allografts were not destroyed *in vitro* by the relevant graft-infiltrating cells whereas donor-specific macrophages were readily lysed. Whether this failure was due to difficulties in labelling the parenchymal cells with radioisotope or to an inherent, low susceptibility to cell-mediated lysis cannot be determined from the available data. In any case, the relationship between target cell lysis *in vitro* and the contribution to allograft destruction *in vivo* remains speculative.

The factors which regulate the non-specific inflammatory response are unknown. However, available data suggest that multiple regulatory mechanisms may operate. Examination of the specificity of cytotoxic T cells which

accumulate in two unrelated allografts carried by the same host revealed that each infiltrate contained T cells cytotoxic for both the relevant and partner grafts; only a marginal preference for the relevant graft was demonstrated (Strom *et al.*, 1977; Ascher *et al.*, 1979). These findings indicate that graft alloantigen *per se* is probably not the major factor influencing the localization of T cell effectors. Häyry and co-workers (1979) used the incorporation of tritiated thymidine as a monitor for cell proliferation in rats carrying kidney allografts. They reported a marked proliferation of lymphoid cells and to a lesser extent, monocytes in rejecting kidney parenchyma during the first week after transplantation. This response exceeded the peak and duration of the specific cellular proliferation in spleen and blood. Thus, cell proliferation *in situ* may help to amplify the inflammatory reaction. The high frequency of Fc-receptor-bearing cells in the infiltrates of rejecting human kidney allografts (Strom *et al.*, 1975; von Willebrand and Häyry, 1978) together with reports of IgG deposits (Carpenter *et al.*, 1976) suggest that antigen-antibody complexes may contribute to the entrapment of Fc-receptor-positive cells at the rejection site. Hoffman and colleagues (1979) reported some evidence indicating that the cell-free eluate expressed from rejecting sponge matrix allografts contained a factor which was chemotactic *in vitro* for both sensitized and naive lymphocytes. This finding suggested that the migration of lymphocytes and possibly other inflammatory cells may be regulated by chemotaxis. Furthermore, it was proposed that such a process may be lymphokine-mediated (Ascher *et al.*, 1979).

This notion is supported by a report by Hattler *et al.* (1973) that lymphocytes recovered from a rejecting human kidney graft released a number of lymphokine activities *in vitro*. These included chemotactic factors for neutrophils and monocytes, mitogenic factor for lymphocytes, migration inhibition factor (MIF) and a procoagulant activity. Indeed, lymphokines play a major role in regulating the inflammatory processes occurring in delayed type hypersensitivity reactions (Adelman *et al.*, 1979).

As mentioned previously, there is good evidence indicating that the process of graft rejection is T cell-mediated (Section 1.4.1). However, there is a need to distinguish between the capacity of T cells to trigger graft rejection and to act as effectors in graft destruction. Evidence outlined here has demonstrated the potential for the latter function. There is also an abundance of literature describing the regulatory functions of T cells expressed *in vitro*. T cells activated either by alloantigen or mitogen release a variety of lymphokine activities including macrophage migration inhibition factor (MIF), mitogenic factor (MF), mononuclear cell chemotactic factor (MCF), macrophage activation factor (MAF), colony stimulating factor (CSF), and T cell replacing factor (TRF) (Rosenstreich and Wahl, 1979). Whether these activities function as inflammatory mediators in the allograft rejection reaction, as the evidence suggests for delayed hypersensitivity, is unknown. Nevertheless, this notion offers an attractive explanation for the mandatory requirement for T cells to procure graft rejection.

1.4.3 The role of antibody in graft rejection

Following early failures to show passive transfer of transplantation immunity to naive recipients using immune serum, it was generally accepted that antibody played little role in the rejection of solid tissue allografts (Section 1.2.2). This premise persisted up until the mid-1960s when it was diagnosed that the "hyperacute" rejection of human kidney allografts was attributable to the presence of pre-existing anti-donor antibodies in the serum of the transplant recipients (Kissmeyer-Nielsen *et al.*, 1966; Williams *et al.*, 1968). This reaction was characterized by an extremely rapid onset, usually within minutes or hours following vascular anastomosis, and the absence of mononuclear cell infiltration in histological sections of the graft tissue. The accumulation of polymorphonuclear leukocytes within the graft vasculature during the early rejection period, followed later by evidence of fibrin deposition and thrombosis, indicated that graft failure was due to vascular obstruction (Kissmeyer-Nielsen *et al.*, 1966; Williams *et al.*, 1968; Milgrom, 1977). These properties clearly distinguished the rejection mechanism from the conventional cell-mediated immune processes.

Follow-up studies in rodent transplantation models confirmed the phenomenon of antibody-mediated rejection by passively transferring donor-specific antiserum to immunosuppressed hosts carrying either intact allografts or xenografts. The sensitivity of foreign grafts to antibody-mediated attack was shown to depend on the nature of the tissue grafted and the post-transplant time at which the antiserum was administered. The hyperacute rejection response

to skin allografts in rats and mice was evoked providing the alloantiserum was administered between days 5 to 10 and days 10 to 16 after transplantation, respectively (Gerlag *et al.*, 1975; Jooste and Winn, 1975). Treatment at earlier times resulted in enhanced allograft survival (Gerlag *et al.*, 1975); at later intervals after transplantation, the grafts were insensitive to attack (Jooste and Winn, 1975). A similar waning of sensitivity was reported for rat skin xenografted to mice (Burdick *et al.*, 1979). In contrast, rat heart xenografts were susceptible to antibody-mediated destruction immediately following transplantation and remained sensitive thereafter (Burdick *et al.*, 1979). Taken together, these findings suggested that vascular endothelium of donor origin represented the target for antibody-mediated attack. The long-standing sensitivity of immediately vascularized heart grafts was consistent with this notion. The initial resistance of skin grafts was tentatively explained by the time required for the anastomosis of donor and host blood vessels, and hence the establishment of a functioning vascular network by which the antibody could gain access to donor antigens (Jooste and Winn, 1975; Gerlag *et al.*, 1975). Furthermore, it seemed reasonable that the resistance of long-established skin grafts was due to the eventual replacement of donor endothelium by host endothelial cells (Burdick *et al.*, 1979). More recent reports have provided conclusive evidence to support this proposal (see Chapter 7). Thus, these findings reinforce the clinical evidence for an antibody-mediated rejection mechanism directed against graft vascular endothelium.

Further studies have demonstrated that antibody-mediated hyperacute rejection is complement-dependent. The hyperacute rejection of kidney and skin allografts in rodents was observed only when the hosts were treated with an exogenous source of active complement (French, 1972; McKenzie and Henning, 1978). These findings suggest that in at least some strains of rats and mice, the fixation of native complement is insufficient to service this rejection process. Winn and colleagues (1973) showed that the acute destruction of rat skin xenografts in mice was inhibited if the hosts were treated with non-complement-fixing anti-donor antiserum or $F(ab')_2$ antibody fragments, or pretreated with cobra venom factor to deplete circulating complement levels. Moreover, the response was mitigated in mice genetically deficient in some complement components. Interestingly, the role of complement appears to be unrelated to antibody-mediated cytotoxicity. This conclusion is based on the failure to observe hyperacute rejection when complement was present and the circulating level of polymorphonuclear cells was depleted by the administration of nitrogen mustard or specific antiserum (Winn *et al.*, 1973). Thus polymorphonuclear cells, which feature prominently in the histologic appearance of the intravascular inflammatory reaction, appear to represent an obligatory component in antibody-mediated graft destruction.

These findings have suggested that the process of antibody-mediated rejection is initiated by the complexing of antibody to donor antigens presented on vascular endothelial cells. In mice, this reaction is directed against class I antigens (McKenzie and Henning, 1978). A reasonable hypothesis is that the activation of the complement cascade by

these immune complexes results in the local production of chemotactic factors which cause polymorphonuclear leukocytes and probably other inflammatory cells to localize within the graft vasculature. It is envisaged that graft necrosis results from primary damage to the vascular bed (Winn *et al.*, 1973).

It is evident that, under normal conditions *in vivo*, immediately vascularized allografts represent the most likely targets for humoral rejection. The extent to which humoral immunity contributes to graft destruction is likely to depend on the presence of pre-existing anti-donor antibodies, or cross-reactive antibodies, and of an active host complement system. Nevertheless, in the absence of these conditions, anti-donor antibody may contribute to graft rejection via the process of antibody-dependent cell-mediated cytotoxicity which does not require complement fixation (Section 1.4.2).

1.5 REQUIREMENTS FOR LYMPHOCYTE ACTIVATION

According to Medawar's dogma, recognition of foreign antigen alone provided the stimulus required for lymphocyte activation (Section 1.2.3). However, over the last decade, a considerable body of evidence has indicated that such an assumption is no longer tenable. This section reviews the evidence provided by studies of graft-versus-host (GVH) reactions and the requirements for T cell activation *in vitro*.

1.5.1 Graft-versus-host reactions

Studies of the local GVH reaction generated in rats by the inoculation of immunocompetent parental (P_1) lymphoid cells under the kidney capsule of genetically tolerant

($P_1 \times P_2$) F_1 hybrid hosts, indicated that the pathogenesis of the GVH lesion was not a consequence of contact between donor lymphocytes and alloantigen presented on the surface of kidney parenchymal cells (Elkins and Guttman, 1968). This conclusion was based on the following observations:

- (i) The GVH response was inhibited when hosts were made leukopenic by total body irradiation, prior to lymphocyte grafting (Elkins, 1966). This finding suggested that the induction of GVH reactivity was dependent on the availability of radiosensitive host leukocytes. Moreover, it was evident that contact with foreign antigen on radioresistant kidney parenchymal cells was an insufficient stimulus for the activation of donor lymphocytes. These conclusions were supported by the failure of local irradiation to eliminate the GVH response (Elkins, 1971).
- (ii) Parental (P_1) lymphocytes transferred beneath the capsule of an isogeneic P_1 kidney grafted to an F_1 host also produced a local GVH-type reaction (Elkins and Guttman, 1968). However, in this situation, a specific immune response was precluded. Consequently, the tissue damage was envisaged as a by-stander effect of an interaction between donor lymphocytes and host leukocytes perfusing the grafted kidney.

Thus, GVH reactions were seen to involve an immune interaction between donor lymphocytes and host leukocytes. However, the demonstration of non-specific tissue damage suggested that a non-specific inflammatory process represented a potential component of the conventional GVH response.

The chick embryo has been used as an alternative model for examining the requirements for GVH reactivity. In this system, the embryo host is immunologically incompetent and GVH reactions are routinely generated by the inoculation of allogeneic adult lymphoid cells (Simonsen, 1962). However, in our laboratory, Lafferty and Jones (1969) failed to observe GVH responses when lymphoid cells from unrelated birds, e.g. pigeon, or from mammals, e.g. sheep, were used for the inoculum. The non-reactive state persisted even though the xenogeneic cells survived and were capable of expressing normal immune reactivity to allogeneic lymphoid cells which had been inoculated into the same embryo host. These findings clearly indicated that contact with foreign antigen alone was not responsible for initiating GVH reactions in the chick embryo. Moreover, the data suggested that there was a species-specific component in the activation process.

Additional studies in the chick embryo demonstrated that such transplant reactions involved an interaction between foreign lymphoid cells and haematogenous blood cells in the embryonic target tissue (Lafferty and Jones, 1969). A GVH-type situation was simulated by equipping embryos with isogeneic adult lymphoid cells and allografting embryonic spleen, liver or bone onto the chorioallantoic membrane. These allografts were readily rejected. However, pretreatment of embryonic spleen and bone by gamma irradiation, and

perfusion of the heart allografts with medium prior to transplantation, protected the graft tissue from destruction. A significant finding was that pretreated heart allografts were rapidly rejected if they were perfused prior to transplantation with embryonic spleen cells of the same donor specificity. Thus, recognition of alloantigen on tissue allografts alone was not responsible for activating the transplant reaction. Instead, graft destruction appeared to result from an interaction between foreign immunocompetent lymphocytes and haematogenous elements in the target tissue. This explanation offered some insight into why the pathological changes induced by conventional GVH reactions in the chick embryo were confined to haemopoietic tissues (Lafferty *et al.*, 1972).

Clearly, these GVH studies provided early evidence that something other than antigen recognition was involved in lymphocyte activation.

1.5.2 *In vitro* studies of T cell activation

The complexity of allograft reactions *in situ*, coupled with the limited scope for experimental manipulation, prompted the development of *in vitro* systems in which to examine the process of lymphocyte activation.

Early studies demonstrated that when two populations of allogeneic lymphocytes were mixed in culture (MLC), the cells underwent blast transformation and proliferated (Bain *et al.*, 1964; Bach and Hirschhorn, 1964). Subsequently, one-way MLCs were set up by using either genetically tolerant F_1 lymphocytes as the stimulating population and parental (P_1 or P_2) lymphocytes as responders, or by blocking the proliferative

response of one allogeneic lymphocyte population by pretreatment with gamma- or X-irradiation, or Mitomycin C (Kasakura and Lowenstein, 1968; Lafferty and Jones, 1969; Häyry *et al.*, 1972). By using this technique, sensitized lymphocytes were shown to lyse only target cells carrying the same H-2 specificity as the stimulating cell population (Häyry and Defendi, 1970). The dual demonstration of a recognition response and effector activity was taken as an indication that the MLC reaction represented an *in vitro* correlate of the process of allograft rejection (Häyry and Defendi, 1970).

As outlined previously (Section 1.4.2), graft rejection is a complex immune mechanism, probably involving a battery of inflammatory cell responses. For this reason, the mixed lymphocyte culture represented an oversimplified counterpart. Nevertheless, it provided a convenient system in which to analyse the requirements for lymphocyte activation. Cytotoxic lymphocytes sensitized *in vivo* or *in vitro* were shown to express the θ alloantigen and hence were identified as thymus-derived T cells (Cerottini *et al.*, 1970; Wagner *et al.*, 1972). Thus, T lymphocytes were recognized as potentially important mediators of cellular immunity. However, implicit in the interpretation of these studies was the concept that recognition of foreign antigen initiated the process of T cell activation.

There is now good evidence that contact with foreign antigen by itself is not sufficient to drive the activation of T cell clones. This conclusion is drawn from the following experimental findings:

1. The capacity to stimulate T cells was shown to be a property of metabolically active cells. Lymphocytes killed by ultraviolet (UV) irradiation failed to activate allogeneic lymphocytes, although they were shown to express histocompatibility antigens (Lindahl-Kiessling and Säfwenberg, 1971; Lafferty *et al.*, 1974; Häyry and Andersson, 1976). Disruption of metabolic integrity by a variety of procedures including freezing and thawing, freeze drying, heating (45°C) and treatment with chemical agents such as urea, acetone, aldehydes and iodo-acetic acid, abolished stimulatory activity without causing obvious damage to cell surface antigens (Hardy *et al.*, 1970; Schellekens and Eijssvoegel, 1970). It should be pointed out here that initial reports of significant stimulation of primary MLCs by subcellular membrane fractions (Wagner and Boyle, 1972; Corley *et al.*, 1975) were subsequently not confirmed (Engers *et al.*, 1975; Häyry and Andersson, 1976; Fast and Fan, 1978). The reason for these discrepancies is not understood.
2. Cell viability *per se* did not distinguish stimulator cell status. Antigen-bearing non-lymphoid cells such as erythrocytes, fibroblasts or cultured epithelial tumour cells failed to stimulate allogeneic lymphocytes (Hardy and Ling, 1969; Greineder and Rosenthal, 1975; Talmage *et al.*, 1977). Polymorphonuclear leukocytes were also inactive (Greineder and Rosenthal, 1975).

3. The process of T cell activation showed phylogenetic restriction; little or no response was generated against xenogeneic leukocytes obtained from phylogenetically unrelated donors (Lafferty and Jones, 1969; Wilson and Nowell, 1970; Greineder and Rosenthal, 1975; Lafferty *et al.*, 1978). However, the species-specific reactivity was not absolute since proliferative responses were demonstrated when the stimulating and responding cell populations were derived from closely related species (Main and Cole, 1966; Woolnough *et al.*, 1979).

Taken together, these findings indicated that the mechanism of T cell activation involved the presentation of foreign antigen on the surface of a metabolically active lymphoid cell, in conjunction with a species-specific component.

1.5.3 Theoretical models for the mechanism of T cell activation

In 1970, Bretscher and Cohn propounded their theory of self-nonsel self discrimination and the induction of the humoral response. In simple terms, the theory proposed that two signals were required for lymphocyte activation. In the following years this concept was adopted by Lafferty and Cunningham (1975) as the basis for a theoretical model of the mechanism of T cell activation.

In terms of the Lafferty model, T cell activation was a two-signal process which required the presentation of foreign

antigen (Signal 1) in combination with an inductive stimulus (Signal 2), later called the lymphocyte costimulator (Lafferty and Cunningham, 1975; Lafferty *et al.*, 1978). This concept suggested the participation of a stimulator (S^+) cell which was capable of providing both signals. Experimentally, cells of lymphoreticular origin expressed the S^+ phenotype, provided their metabolic integrity was preserved (Lafferty and Cunningham, 1975; Talmage *et al.*, 1977). Antigen-bearing non-lymphoid cells and S^+ cells rendered metabolically inactive by UV-irradiation failed to stimulate allogeneic lymphocytes and therefore were designated S^- cells. An important finding was that this inability was rectified by supplementing the mixed cell cultures with the supernatant obtained from Con A-activated spleen cells (Talmage *et al.*, 1977; Lafferty *et al.*, 1978). This demonstration provided early experimental evidence for the existence of a lymphocyte costimulator (Signal 2). Moreover, the presence of costimulator by itself was insufficient to stimulate T cell activation. Both the presentation of foreign antigen on the surface of S^- cells together with a source of costimulator were obligatory for the initiation of the activation process. Thus, the experimental evidence supported the two-signal model of T cell activation. In addition, these findings suggested that the inactivation of S^+ cells by UV-irradiation was attributable to the cellular metabolism required for the production and/or release of the costimulator (Lafferty *et al.*, 1978).

The support for this two-signal model was strengthened when it was shown that costimulator activity was species-specific (Lafferty *et al.*, 1978; Woolnough *et al.*, 1979).

Following the demonstration of a normal precursor frequency for xenoreactive T cells, the phylogenetic restriction in the mechanism of T cell activation was attributed to the species-specificity of the lymphocyte costimulator (Woolnough *et al.*, 1979). In other words, species compatibility between the donors of the costimulator and responding lymphocytes was required for the expression of xenoreactivity.

Subsequently, Bach and co-workers (1976) proposed an alternative mechanism for T cell activation. Their model for the induction of the cytotoxic T cell response distinguished a functional role for different T cell subsets. In brief, the activation process required two signals; the first signal was provided by the recognition of class I alloantigens presented on the stimulating cell, by the cytotoxic T cell precursor. This signal induced the differentiation of the precursor cell to a "poised" cytotoxic cell. A separate population of T cells (helper T cells) activated solely by the recognition of class II antigens also expressed on the surface of the stimulating cell, then provided the second signal or "help" required to transform the poised cytotoxic cell into the fully differentiated T cell effector.

Thus, the Bach model postulated a dichotomy in the requirements for the activation of T cells by class I and class II alloantigens; although one signal was required to activate helper T cells, two signals were necessary for the activation of cytotoxic T cells. In contrast, the Lafferty model made no such distinction. Recent evidence demonstrating that helper T cells also require two signals for activation, will be outlined in Chapter 7.

Several experimental observations have not supported another basic assumption of Bach's model; that the recognition of class II alloantigens provides the second signal required for the induction of a cytotoxic T cell response. Gamma-irradiated P815 mastocytoma tumour cells and gamma-irradiated EL-4 lymphoma cells, which do not express class II antigens, have been shown to generate strong cytotoxic T cell responses (Lafferty *et al.*, 1978). Similarly, cytotoxic T cells have been demonstrated in MLCs where the stimulating and responding populations differed in only class I antigens (Bach and Alter, 1978). These discrepancies led Bach to postulate that an alternative pathway for T cell activation operated in the presence of only class I antigenic differences (Bach and Alter, 1978; Bach *et al.*, 1979). Nevertheless, this provision did not explain the failure of UV-irradiated allogeneic spleen cells, which carry both class I and class II antigens, to stimulate a cytotoxic response (Lafferty *et al.*, 1974).

Using an *in vivo* system for sensitization, Batchelor *et al.* (1978) also concluded that two signals were required for the activation of cytotoxic T cells. They immunized mice with membrane fragments carrying class I alloantigens in the presence or absence of class II alloantigens, and showed that in both situations the host lymphocytes failed to demonstrate cytotoxic activity when tested *in vitro* against donor-specific targets. In contrast, immunization with heavily irradiated, viable allogeneic lymphoid cells stimulated a strong cytotoxic response. These findings suggested that the second signal was provided by a viable lymphoid cell. Furthermore, positive

cytotoxic responses obtained using EL-4 lymphoma cells as immunogens supported Lafferty's observations that class II antigens did not provide the second signal.

1.6 THE BARRIER TO ALLOTRANSPLANTATION

1.6.1 A theoretical approach

From a theoretical viewpoint, the various models for the pathway of T cell activation provide a clue to the mechanism of graft rejection, a T cell-dependent process (Section 1.4.1). Each model discussed in the previous section (Section 1.5.3) shares the common postulate that lymphoid cells or "passenger leukocytes" carried passively within a tissue transplant constitute the major source of tissue immunogenicity (Lafferty and Talmage, 1976; Sollinger and Bach, 1976; Batchelor, 1978).

According to the Bach model, passenger leukocytes are immunogenic because they provide the class II alloantigens necessary for helper T cell induction (Sollinger and Bach, 1976). However, in view of the *in vitro* evidence against the one-signal model for helper T cell activation (Section 1.5.3) and the rejection of tissue allografts in the presence of only class I antigenic disparities (Klein, 1972; McKenzie and Snell, 1973; Klein *et al.*, 1976) this theory will not be considered further here in relation to tissue transplantation. The Lafferty and Batchelor models both see passenger leukocytes as the only cells of graft origin that are capable of providing both signals required for T cell activation and the generation of a graft-specific T cell response. In other

words, these two models agree that MHC alloantigens *per se* are weak T cell immunogens and are only rendered immunogenic when presented on the surface of metabolically active lymphoreticular cells (Lafferty and Woolnough, 1977; Batchelor, 1978). Nevertheless, there appears to be an inconsistency in Batchelor's model. Although class II alloantigens are not envisaged as the second signal, Batchelor maintains that helper T cells are required to generate a strong graft-specific response (Batchelor, 1978). Implicit in this hypothesis is the need for graft stimulator cells to carry class II antigens. In view of this confusion, the Lafferty model presents a more favourable and explicit prediction for the role of passenger leukocytes in graft rejection.

According to the Lafferty model, blood cells carried within the graft, and not transplantation antigen *per se*, constitute the major barrier to allotransplantation (Lafferty and Talmage, 1976; Lafferty and Woolnough, 1977). In compliance with the model's terminology, parenchymal graft cells express the S^- phenotype and lymphoreticular cells present in the graft either as passenger leukocytes or fixed tissue components represent a rich source of S^+ cells. The theory postulates that donor blood cells in the graft can contribute to graft destruction by both induction and effector mechanisms. Once activated against graft-specific alloantigens on donor leukocytes, host T cells may contribute directly to the rejection process via cytotoxic activity, or indirectly by recruiting non-specific inflammatory cells of host origin to the rejection site, and inducing their activation and proliferation (Section 1.4.2). Similarly, the

local release of lymphokine(s) by activated T cells following alloantigen recognition may influence the activation and proliferation of responsive blood cells of donor origin. It is conceivable that *in situ* such responses could lead to local, non-specific damage to the graft and thereby contribute to the inflammatory component of the rejection response (Lafferty and Talmage, 1976; Lafferty and Woolnough, 1977). Thus, donor blood cells carried within a tissue transplant may play dual roles in the process of allograft rejection.

Consequently, this theory predicts that pretreatment of graft tissue to remove blood cells prior to transplantation should facilitate allograft survival. As mentioned previously, pioneer studies in the early 1930s hinted that the maintenance of graft tissue *in vitro* prior to transplantation was beneficial (Section 1.1). Supportive evidence for successful allotransplantation following the pretreatment of donor tissue was provided by the studies of GVH-type reactions in the chick embryo (Section 1.5.1). Nevertheless, the notion of modifying allograft immunogenicity represents a blatant digression from the traditional view that host immunosuppression and tissue-typing constitute the only logical solutions to allograft rejection (Section 1.2.3). Interestingly, the theory provides a rational explanation for Medawar's observation that mouse leukocytes but not erythrocytes were capable of invoking transplantation immunity (Section 1.2.1; Section 1.2.3; Medawar, 1946). Indeed, early genetic studies of tumour transplantation by Gorer *et al.* (1948) had indicated that mouse erythrocytes carried H-2 antigens.

1.6.2 The passenger leukocyte concept

The idea that passenger lymphoid cells played an instrumental role in graft rejection was originally advocated by Snell (1957b). This proposal was based on the notion that donor lymphocytes carried within graft tissue acted as vectors which could rapidly transport foreign antigen to the regional lymph nodes, the induction site for the allograft response (Snell, 1957b). However, the experimental evidence now available does not support this argument. Allogeneic sheep lymphocytes, rendered metabolically inactive by pre-treatment with Mitomycin C and inoculated *in vivo* via the subcutaneous route, generated a barely detectable immune response in the regional popliteal lymph node, even though the cells expressed surface alloantigens (Scolley *et al.*, 1974). This demonstration indicated that the role of lymphoid cells in generating alloresponses does not reside solely in the capacity to deliver antigen to the regional lymphoid tissues. Moreover, Strober and Gowans (1965) demonstrated effective sensitization of P_1 rat lymphocytes following *in vivo* or *in vitro* perfusion through a ($P_1 \times P_2$) F_1 kidney. Thus, the apparent sensitization within the simulated graft environment pre-empted an obligatory role for the regional lymph nodes as the site for lymphocyte activation.

However, the basic concept of a role for passenger leukocytes in graft rejection was revived when Steinmuller (1967, 1969) showed that allogeneic leukocytes carried within a skin graft were highly immunogenic. Skin isografts taken from strain "A" mice rendered neonatally tolerant of strain "B" and transplanted to naive "A" mice immunized the latter

against strain "B" skin allografts (Steinmuller, 1967). Since the tolerant donors were haemopoietic chimeras, the phenomenon was explained by the passive transfer of allogeneic leukocytes via the primary skin isograft. In support of this proposal, the immunizing capacity of such skin isografts was shown to be a function of the number of leukocytes present and the degree of leukocyte chimerism of the donors (Steinmuller, 1969). Unlike the passive function proposed by Snell, these studies suggested a potentially active role for passenger leukocytes in triggering allogeneic interactions.

Similarly, studies of GVH reactions in the rat kidney model (Section 1.5.1) suggested that passenger leukocytes played an important role in the rejection of organ allografts (Elkins and Guttman, 1968). Support for this notion was provided by investigations of GVH-type reactions in the chick embryo; it was demonstrated that the removal of blood cells from graft tissue prior to transplantation successfully prolonged allograft survival (Section 1.5.1). This finding reinforced earlier predictions that pretreatment of transplant tissue to remove passenger leukocytes could be an important step in overcoming the clinical problem of graft rejection (Elkins and Guttman, 1968).

Consequently, attention turned towards the objective of eliminating passenger leukocytes from graft tissue by pre-treating donors with agents that induced leukopenia. Such procedures included whole body irradiation and the administration of cyclophosphamide or anti-lymphocyte xenosera. However, this approach only marginally prolonged the survival of rat kidney and skin allografts transplanted

across a major histocompatibility barrier (Guttmann *et al.*, 1967; Stuart *et al.*, 1971; Kyger and Salyer, 1973) and rat heart allografts carrying multiple minor histocompatibility differences (Freeman *et al.*, 1971). There was also a lack of correlation between the degree of donor leukopenia and graft survival (Freeman *et al.*, 1971). Stuart and colleagues (1971) attempted to remove passenger leukocytes by first allografting rat kidneys to passively enhanced intermediate hosts. At 60 to 300 days post-transplantation, the kidney grafts were retransplanted to naive recipient rats isologous to the intermediate hosts. However, even by taking these measures to ensure donor leukocyte depletion, only a delay in the onset of rejection was achieved. Together, these findings questioned the relative importance of the role for passenger leukocytes in activating the allograft rejection response. Interestingly, a more recent report has indicated that the delayed rejection of secondary transplants of enhanced rat kidney allografts may be attributable to an antibody-mediated mechanism (see Chapter 7).

On the other hand, the presence of allogeneic passenger leukocytes was shown to be a sufficient stimulus to generate an allogeneic reaction. In contrast to isograft controls, the majority of rat kidney isografts taken from chimeric donors failed to function following transplantation. This finding was attributed to non-specific kidney damage resulting from the interaction between allogeneic passenger leukocytes and host lymphoid cells (Stuart *et al.*, 1971). Steinmuller and Hart (1971) demonstrated that chimeric mouse skin allografts which carried non-H-2 alloantigens on parenchymal cells and isogeneic passenger leukocytes were significantly less

effective than control allografts in eliciting transplantation immunity. However, this inefficacy was evident only during the first four days after transplantation. Thereafter, the immunizing capacity of the skin graft markedly improved. Similar findings were subsequently reported for irradiated rat skin allografts transplanted across major histocompatibility barriers (Kyger and Salyer, 1973). These reports suggested that allogeneic passenger leukocytes played an important immunogenic role immediately following transplantation. However, it was apparent that the residual tissue immunogenicity was due to some other component which appeared to operate after graft revascularization (Kyger and Salyer, 1973). Subsequent *in vitro* studies suggested that the Langerhans cell population represents the fixed immunogenic component of skin (Stingl *et al.*, 1978).

Thus, early attempts to improve allograft survival by reducing or removing the passenger leukocyte population prior to transplantation were unsuccessful. This failure led to some confusion over the extent to which passenger leukocytes contributed to tissue immunogenicity (Billingham, 1971). It is now apparent that the main reason for these difficulties can be attributed to the choice of tissue for transplantation. This argument will be elaborated upon later in relation to the data presented in this thesis (see Chapter 7). Nevertheless, the clinical success obtained with renal allografts taken from cadaver donors pretreated with cyclophosphamide and methylprednisolone helped to foster the passenger leukocyte concept (Guttmann *et al.*, 1975).

1.6.3 The organ culture technique

The technique of pretreating donor tissue *in vitro* was re-introduced into transplantation research when Jacobs and Huseby (1967, 1968) demonstrated prolonged survival of Leydig tumour cell allografts following *in vitro* culture. Although the rejection of cultured tumour allografts by sensitized hosts indicated that the cultured tissue still carried surface alloantigens, the recovery of tissue immunogenicity by passage through isologous hosts was taken as an indication that the pretreatment *in vitro* repressed antigen expression (Jacobs and Huseby, 1967). Ironically, the impact of this tissue culture technique was not felt until Summerlin and colleagues (1973) reported the prolonged survival of human and mouse skin allografts following cultivation of the donor tissue *in vitro*. However, these findings were subsequently not confirmed in the same laboratory and consequently the original work was discredited (Ninneman and Good, 1974). Nevertheless, independent groups reported significant prolongation in the survival of allografts of mouse ovary (Jacobs, 1974; Lueker and Sharpton, 1974) and thyroid (Lafferty *et al.*, 1975) following organ culture prior to transplantation. Together, these reports firmly re-established the organ culture procedure in the repertoire of techniques designed to hurdle the transplantation barrier (Billingham, 1976).

The organ culture technique has served as a particularly useful tool in studies of the mechanism of graft rejection. Lafferty and co-workers (1976a, 1976b) showed on the basis of histological and functional criteria, that long-term organ culture of mouse thyroid in a gas phase of 95% O₂, 5% CO₂

indefinitely prolonged allograft survival. The cultured thyroid allografts were rejected if the recipient mice received a single intravenous injection of 10^5 viable peritoneal cells of the same donor specificity, at the time of transplantation (Lafferty *et al.*, 1976b; Talmage *et al.*, 1976). This finding clearly demonstrated that the cultured tissue carried recognizable antigens. Consequently, the effect of *in vitro* culture was not attributable to antigenic modulation. Non-viable donor peritoneal cells and peritoneal cells of recipient strain specificity were ineffective in activating the allograft rejection response (Lafferty *et al.*, 1976b; Talmage *et al.*, 1976). On this basis, it was suggested that organ culture reduced graft tissue immunogenicity by removing passenger leukocytes prior to transplantation. Further support for this interpretation was provided by the demonstration that pretreatment of thyroid donors with cyclophosphamide markedly decreased the duration of culture required to achieve successful allotransplantation (Lafferty *et al.*, 1976a). In contrast to the effects of organ culture, cyclophosphamide pretreatment did not destroy the vascular network of the donor tissue (Lafferty and Woolnough, 1977). Thus, the prolonged survival of a proportion of uncultured thyroid allografts obtained from cyclophosphamide-pretreated donors argued against a role for vascular endothelium as a source of stimulation for the allograft rejection response (Lafferty and Woolnough, 1977). Furthermore, the differential rejection rate of cultured and uncultured thyroid allografts transplanted to the same host provided suggestive evidence for

a contribution of donor blood cells to the non-specific inflammatory component of graft rejection (Section 1.6.1). Uncultured allografts were rejected more acutely and, at the histological level, the inflammatory response was much more vigorous than in the case of the cultured grafts (Lafferty and Woolnough, 1977).

The experimental evidence documented on the effect of organ culture on thyroid allotransplantation was compatible with the theoretical prediction that treatment of donor tissue to remove blood cells before transplantation should facilitate allograft survival and thereby dismiss the need for host immunosuppression (Section 1.6.1). The technique gained further support when Bach's group reported the long-term survival of cultured rat thyroid xenografts in mice (Sollinger *et al.*, 1977). Talmage and Dart (1978) provided some insight into the possible mechanism operating to reduce tissue immunogenicity when they demonstrated that the survival of short-term cultured mouse thyroid allografts was significantly improved by increasing the oxygen tension of the culture gas phase. This finding supported the notion that the high oxygen atmosphere selectively destroyed passenger leukocytes (Lafferty *et al.*, 1976b). In addition, it offered a reasonable explanation for the failure of Raaf and colleagues (1976) to demonstrate improved survival of rat thyroid allografts following organ culture in a 5% CO₂, 95% air gas phase.

Thus, organ culture of donor tissue prior to allotransplantation can markedly reduce tissue immunogenicity and facilitate allograft survival. In contrast to Medawar's fears (Section 1.2.3), this effect is achieved without alteration to alloantigen expression. There is suggestive

evidence that the immunogenic modulation is due to loss of passenger leukocytes. Consequently, the success of the organ culture technique has consolidated the passenger leukocyte concept of graft rejection.

1.7 THE RELEVANCE OF THIS THESIS TO CLINICAL PANCREATIC ISLET TRANSPLANTATION

It is now evident that treatment of diabetes mellitus with daily administration of exogenous insulin does not prevent the development of severe microangiopathic disorders which can ultimately cause blindness, renal dysfunction, cardiac disease and gangrene. The postulate that these secondary complications result from inadequate control of carbohydrate metabolism has provided the impetus for clinical pancreatic islet tissue transplantation (Connolly *et al.*, 1973; Broe *et al.*, 1981; Sutherland, 1981a). However, efforts to attain physiological replacement of endocrine function using whole or segmental pancreas for transplantation have met with little success (Broe *et al.*, 1981; Sutherland, 1981b). This failure has been attributed to technical difficulties, including the drainage of exocrine enzymes, pancreatic fibrosis and vascular thrombosis, and to the immunological problem of allograft rejection (Sutherland, 1981b). In response to the former, attention has turned towards isolated adult islets and the foetal pancreas as alternative sources of tissue for transplantation. However, the adult human pancreas is very fibrous and consequently there have been difficulties in isolating sufficient numbers of islets for transplantation (Najarian *et al.*, 1975; Ferguson *et al.*, 1977; Yasunami

et al., 1981). Even with the use of unpurified, dispersed pancreatic tissue, allograft rejection remains the major obstacle (Sutherland, 1981b). Likewise, the failure of foetal pancreas allografts is probably attributable to immunological rejection (Valente *et al.*, 1980; Groth *et al.*, 1980; Usadel *et al.*, 1980). Clinicians are also faced with the dilemma that high dosage steroids commonly used in immunosuppressive regimens are diabetogenic and therefore are unsuitable for the treatment of diabetic patients (Broe *et al.*, 1981).

It is evident, therefore, that methods of reducing the immunogenicity of pancreatic islet tissue prior to transplantation would be of potential clinical value.

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

All animals were obtained from the Animal Breeding Establishment at the John Curtin School of Medical Research. Inbred mice had access to water and were fed *ad libitum* with Actamul mouse and rat cubes (Pfizer Chemicals, Australia).

BALB/c fetuses at 17 days of gestation were used as donors of mouse foetal pancreas. To obtain 17 day-old BALB/c fetuses, BALB/c breeding pairs were set up; adult male BALB/c mice were housed individually with a single adult female mouse. Mating was monitored by daily inspection of female mice for the presence of a vaginal plug. The day on which a plug was identified was designated day 0. At 17 days, pregnant mice were sacrificed by cervical dislocation and the fetuses were removed by hysterotomy under sterile conditions.

Thyroid tissue and skin for transplantation were obtained from male BALB/c mice, 9 to 12 weeks old. Isolated adult islets were prepared from male BALB/c donors, 8 to 10 weeks old.

Male BALB/c (H-2^d) mice were used as isograft recipients and male CBA (H-2^k) mice were used as recipients of allografts bearing major plus minor histocompatibility differences. Transplant recipients were 9 to 12 weeks old. Male DBA/2 (H-2^d) mice, 10 to 15 weeks old, were used as recipients of allografts carrying only non-H-2 differences.

2.2 CULTURE MEDIA

RPMI 1640 medium (pH 7.4; Microbiological Associates) was prepared by dissolving 10.4 gm of medium powder in 1 litre of double distilled, deionized water supplemented with 1.0gm of sodium bicarbonate.

Eagle's Minimal Essential Medium (EMEM) was prepared by dissolving 10.0gm of medium powder (F-15, GIBCO) in 1 litre of double distilled, deionized water supplemented with 2.2gm of sodium bicarbonate.

RPMI 1640 and EMEM were supplemented with antibiotics (penicillin, 100 units/ml, streptomycin, 100 μ g/ml, and neomycin, 100 μ g/ml) and sterilized by filtration through a 0.22 μ filter (Millipore Corporation). From 2 weeks after preparation, RPMI 1640 medium was supplemented with L-glutamine, 0.3gm/100 ml, immediately before use.

Heat-inactivated foetal calf serum was prepared by incubating selected serum batches (Flow Laboratories, Australia) at 56°C for 30 minutes.

Hanks Balanced Salt Solution (HBSS) was prepared according to the procedure described by Hanks and Wallace (1949). HBSS was sterilized by autoclaving at 112°C for 20 minutes. HBSS was buffered with 0.5gm N-2-hydroxyethyl piperazine-N¹-2-ethanesulfonic acid (HEPES: Sigma)/100 ml immediately before use.

Phosphate Buffered Saline (PBS; calcium and magnesium-free, pH 7.3) was prepared according to the following formula: sodium chloride, 80.0gm; disodium hydrogen phosphate, 12.5gm; monosodium hydrogen phosphate, 4.0gm; distilled water, 1 litre. PBS was sterilized by autoclaving at 121°C for 20 minutes.

Alsevers solution (pH 6.1) was prepared according to the following formula: sodium citrate, 8.0gm; sodium chloride, 4.2gm; glucose, 20.5gm; citric acid, 0.8gm; distilled water, 1 litre. Alsevers solution was sterilized by autoclaving at 112°C for 20 minutes.

Normal saline (0.9% sodium chloride) was prepared by dissolving 9.0gm sodium chloride in 1 litre of distilled water. Normal saline was sterilized by autoclaving at 121°C for 20 minutes.

2.3 ORGAN CULTURE OF MOUSE FOETAL PANCREAS

BALB/c foetuses at 17 days of gestation were sacrificed by decapitation and the foetal pancreas was removed with the aid of a stereomicroscope (Carl Zeiss, West Germany) and placed in HEPES-buffered HBSS (Section 2.2) on ice, using a sterile technique. The mesentery surrounding the pancreas was removed. In initial experiments the foetal pancreas was cultured as a whole organ. Generally, each pancreas was cut into 3 segments to facilitate gas diffusion.

2.3.1 Submerged Culture

Foetal pancreas tissue was submerged in culture medium. Whole foetal pancreases were transferred to 35 mm hydrophobic petri dishes (Falcon 1008), 1 pancreas/dish. Foetal pancreas segments were transferred either to 35 mm hydrophobic petri dishes (Falcon 1008), 2 or 3 segments/dish, or to hydrophobic tissue culture plates (Linbro 76-247-05), 2 or 3 segments/well. Each culture vessel contained 1 ml RPMI 1640 culture medium supplemented with 10% heat-inactivated foetal calf serum (Section 2.2). The tissue was cultured in a humidified gas phase of 95% O_2 , 5% CO_2 at 37°C for 10-24 days. The culture medium was changed three times per week.

2.3.2 Grid Culture

Foetal pancreas tissue was cultured at the gas-medium interface. A sterile, stainless steel triangular grid (60 mesh;

Falcon 3014) was placed in the centre well of each 60 mm double well-petri dish (Falcon 3037). A triangular piece of sterilized lens paper (Kodak) was placed over the grid so that the apices were submerged in culture medium. The lens paper was moistened by diffusion of culture medium. Foetal pancreas tissue was placed on top of the lens paper substrate; 2 whole pancreases/grid or 6 foetal pancreas segments/grid. Each centre well contained 1 ml RPMI 1640 culture medium supplemented with 10% heat-inactivated foetal calf serum (Section 2.2); the outer well contained 2 ml of sterilized distilled water to humidify the immediate culture environment. The tissue was cultured in a humidified gas phase of 95% O₂, 5% CO₂ at 37°C for 10-17 days. The culture medium was changed three times per week.

2.4 PREPARATION OF FOETAL MOUSE PROISLETS

Foetal proislets were isolated from foetal mouse pancreas using a procedure based on the method of Hellerström *et al.* (1979). Foetal pancreases were removed from BALB/c fetuses at 17 days of gestation, using a sterile technique. The surrounding mesentery was removed with the aid of a stereomicroscope (Carl Zeiss, West Germany) and each pancreas was cut into 3 segments. The pancreas segments were transferred to a siliconized vial and digested with 6 mg/ml collagenase (Boehringer Mannheim). To facilitate the digestion process the vial containing the collagenase suspension was agitated in a shaking water bath for 5 minutes at 37°C. The shaking water bath consisted of an oscillating platform, 200 cycles per minute, assembled in a water bath (Anax-Labmaster, Australia)

maintained at 37°C. The collagenase was made up in 2 ml of isolation medium - HEPES-buffered HBSS (Section 2.2) supplemented with BSA (0.15%), DNase (Sigma Type 1, 0.01 mg/ml) and antibiotics (penicillin, 100 units/ml, streptomycin, 100 µg/ml and neomycin, 100 µg/ml) - and sterilized by filtration through a 0.20µ filter (Amicon).

The digest was then shaken vigorously by hand for a further 1½-2 minutes and the digestion step terminated by dilution with excess isolation medium. The digested foetal tissue was then allowed to settle on ice for 10 minutes. The supernatant was removed and the tissue was washed twice with 10-15 ml isolation medium to remove residual collagenase. The digested tissue was resuspended in 15-18 ml isolation medium and stirred for 1 hour at 25°C. The isolation medium was removed (as above) and the digest was suspended in RPMI 1640 culture medium (1 ml/donor foetal pancreas) supplemented with 10% heat-inactivated foetal calf serum (Section 2.2). The suspension of finely digested tissue was transferred to hydrophobic tissue culture plates (Linbro 76-247-05), 2 ml/well, using a siliconized Pasteur pipette and cultured for 4 days in a humidified atmosphere of 5% CO₂, 95% air at 37°C. The culture medium was changed on day 2; 1 ml of medium was removed from each well using a Pasteur pipette and each well was supplemented with 1 ml of fresh culture medium.

2.5 ISOLATION OF ADULT MOUSE ISLETS

Groups of 8-12 donor BALB/c mice were anaesthetized with ether (Hoechst, Australia) and sacrificed by cervical dislocation. The pancreas was removed, finely diced with

scissors and washed in isolation medium (Section 2.4). The minced tissue was allowed to settle at 4°C for 4 minutes and debris remaining in suspension was removed with a Pasteur pipette. After three 30 second-dicings and subsequent washings, the minced tissue was transferred to a siliconized 15 ml graduated glass centrifuge tube and allowed to settle for an additional 4 minutes. The volume of tissue was noted and the tissue was then transferred to a siliconized 20 ml glass vial. Collagenase (Boehringer Mannheim) was freshly prepared in PBS (Section 2.2) at a concentration of 10 mg/ml, and then filtered through a 0.20 μ filter (Millipore Corporation). One ml of this solution was added to each 1 ml volume of minced pancreatic tissue and the volume was made up to 5 ml with isolation medium. The pancreatic tissue was then digested at 37°C for 17 minutes in a shaking water bath (Section 2.4). The digest was vigorously shaken by hand for an additional 15-20 seconds, in order to disperse any residual clumps of tissue. The enzymatic digestion was arrested by the addition of 15 ml of chilled isolation medium and the digest was then allowed to settle for 4 minutes at 4°C. The supernatant was removed, and the digested tissue was washed twice with isolation medium. Individual islets were identified using a stereomicroscope (Olympus SZ-111, Japan) set at 15 x magnification and then removed from the digest with a fine siliconized pipette. The isolated islets were placed on ice in a petri dish containing isolation medium. The islet yield averaged 40 islets per mouse pancreas. At the end of the isolation procedure, the islets were transferred by pipette to a petri dish containing RPMI culture medium supplemented with heat-inactivated foetal

calf serum (Section 2.2). The isolated islets were kept on ice prior to preparation for organ culture.

2.6 ORGAN CULTURE OF ADULT MOUSE ISLETS

Adult islets were cultured as islet clusters. Groups of approximately 50 islets were transferred to 35 mm hydrophobic petri dishes (Falcon 1008) containing 2 ml of RPMI 1640 culture medium supplemented with 10% heat-inactivated foetal calf serum (Section 2.2). To promote islet aggregation, initially each group of islets was gently rotated on an orbital platform (Gallenkamp SGM-300, England) at 50-60 cycles per minute, for 18-24 hours. The cultures were maintained at 37°C in a humidified gas phase of 95% O₂, 5% CO₂. The islet aggregates were then maintained as stationary cultures for 7 days in the same gas phase. The culture medium was changed 3 times per week; on the first and subsequent changes, the culture medium was removed using a Pasteur pipette and 0.75 ml fresh culture medium was replaced in each dish. During the culture period each aggregate condensed into a tight cluster suitable for transplantation.

2.7 ORGAN CULTURE OF MOUSE THYROID

Mouse thyroid lobes were obtained from BALB/c donor mice pretreated with 300 mg/kg cyclophosphamide (Endoxan-Asta, Bristol Laboratories, Australia) intraperitoneally on days -4 and -2. The mice were killed with ether (Hoechst, Australia) on day 0. The thyroid lobes were removed and transferred to 35 mm hydrophobic petri dishes (Falcon 1008), 2 or 3 lobes/dish. Each culture dish contained 1 ml EMEM supplemented with 10%

heat-inactivated foetal calf serum (Section 2.2). The tissue was cultured in a humidified gas phase of 95% O₂, 5% CO₂ at 37°C for 21 days. The culture medium was changed 3 times per week.

2.8 PREPARATION OF FULL-THICKNESS SKIN FOR TRANSPLANTATION

Adult male BALB/c donor mice were sacrificed by cervical dislocation. A small mid-ventral incision was made and the body skin was then peeled from the carcass. The pelt was transferred to a petri dish containing gauze saturated with PBS (Section 2.2). The skin, dermis uppermost, was spread flat on the gauze and kept moist with PBS. Attached muscle was removed by carefully scraping the dermis with a scalpel blade (Size 22; Swann-Morton, England). The prepared body skin was kept on ice prior to transplantation.

2.9 PREPARATION OF PLASMA

Donor mice were anaesthetized with avertin anaesthetic (Section 2.12). The thorax was opened with scissors and approximately 0.5 ml of blood was removed via cardiac puncture, into a 1 ml disposable syringe (Pharmaseal) containing 0.05 ml of 0.1 M sodium citrate. The blood sample was gently mixed with the sodium citrate solution to prevent clotting. The mice were immediately killed by cervical dislocation. The sample was transferred to a microcentrifuge tube (Vetri; 1.5 ml with attached cap; Max Richter, West Germany) and centrifuged at approximately 8,740 g for 4 minutes in a Beckman microcentrifuge. The plasma was removed and diluted 1:1 with 0.9% sodium chloride (Section 2.2). Aliquots of the diluted plasma were stored in plastic microcentrifuge tubes

at -20°C .

2.10 PREPARATION OF PROISLETS FOR TRANSPLANTATION

To facilitate transplantation, foetal proislets were initially embedded in a plasma clot. A suspension of isolated proislets harvested from 2 foetal pancreases (1 culture well of tissue) was transferred to a siliconized glass tube with a V-shaped bottom. The proislets were allowed to settle at room temperature (25°C) and the supernatant was removed. The proislet tissue was then resuspended in approximately $30\ \mu\text{l}$ diluted plasma (Section 2.9), isologous to the transplant recipient. The proislets were allowed to settle and excess plasma was removed until only approximately $10\ \mu\text{l}$ plasma remained. A plasma clot was then prepared by the addition of excess thrombin powder (Parke-Davis). The clot was resuspended in HEPES-buffered HBSS (Section 2.2) and transferred to a petri dish on ice. Plasma clots containing proislets were kept on ice until transplantation.

2.11 PREPARATION OF UNCULTURED ADULT ISLETS FOR TRANSPLANTATION

Loose clusters of uncultured islets were prepared by placing 50 adult BALB/c islets in 35 mm hydrophobic culture dishes (Falcon 1008), each containing 2 ml of RPMI 1640 culture medium supplemented with 10% heat-inactivated foetal calf serum (Section 2.2). The islets were maintained in a gas phase of 5% CO_2 , 95% air at 37°C and rotated on an orbital platform (Gallenkamp SGM-300) at 50-60 cycles per minute for 4 hours, by which time loose clusters of islets had formed.

2.12 PREPARATION OF AVERTIN ANAESTHETIC

Avertin was prepared by dissolving 1.0gm of 2,2,2-tribromoethanol (Fluka AG, Buchs SG, Switzerland) in 1 ml of 2-methyl-2-butanol (Fluka AG, Buchs SG, Switzerland). The solution was diluted to a final volume of 50 ml with hot tap water (50°C) and shaken vigorously to mix. The v/w dose of avertin used to anaesthetize mice was 0.010 - 0.013 ml/gm body weight, administered intraperitoneally.

2.13 TRANSPLANTATION UNDER THE KIDNEY CAPSULE

Recipient mice were anaesthetized with avertin (Section 2.12) and shaved on the left flank with electric animal clippers (Oster, USA) to remove hair from the incision site. Instruments were sterilized by boiling in an instrument sterilizer, (Labmaster, Australia) immediately prior to use. The left flank was swabbed with 70% ethanol and a vertical midline incision was made in the skin using a scalpel blade (Size 11; Swann-Morton, England). The abdominal wall was cut with scissors and the kidney exposed and held in position with a cotton swab. A small incision was made in the kidney capsule using a 19 gauge needle (Terumo, Australia) and a space was made between the capsule and kidney parenchyma, using a rounded 27 gauge Luer lock needle ($\frac{1}{2}$ "; Unimed, Switzerland). The transplant tissue was then inserted under the capsule using the modified Luer lock needle as a probe. In the case of multiple tissue pieces, each piece was positioned at a separate site under the kidney capsule. During the transplantation procedure, the kidney was swabbed regularly with HEPES-buffered HBSS (Section 2.2), to prevent dehydration. The kidney

carrying the transplant tissue was then replaced in the abdominal cavity and the incised skin was sutured together with autoclips (9 mm; Clay Adams, USA). The transplanted mice were placed under an incandescent lamp until they recovered from the anaesthesia.

2.14 ORTHOTOPIC TRANSPLANTATION OF FULL-THICKNESS SKIN

Recipient mice were anaesthetized with avertin (Section 2.12). The graft bed was prepared on the dorsal body surface and measured approximately 1 cm x 1.5 cm. The host skin, comprising the epidermis and dermis, was removed in small strips, using scissors. The panniculus carnosus, a thin layer of striated muscle below the dermis, was left relatively intact. This muscle layer is furnished with a rich vascular network and is retained in order to facilitate revascularization of the grafted skin (Billingham, 1961). The graft bed was swabbed regularly with PBS (Section 2.2) to prevent dehydration. A piece of full-thickness donor skin (Section 2.8) slightly larger in size than the prepared graft site, was positioned over the graft bed. The graft skin was arranged so that the direction of hair growth of the donor skin was counter to the hair growth of the recipient mouse. This procedure facilitated long-term identification of skin isografts (Fig. 2.1). The grafted skin was sutured to the host using autoclips (9 mm; Clay Adams, USA). On day 11 or day 12 post-transplantation, the autoclips were removed to allow subsequent assessment of graft survival.

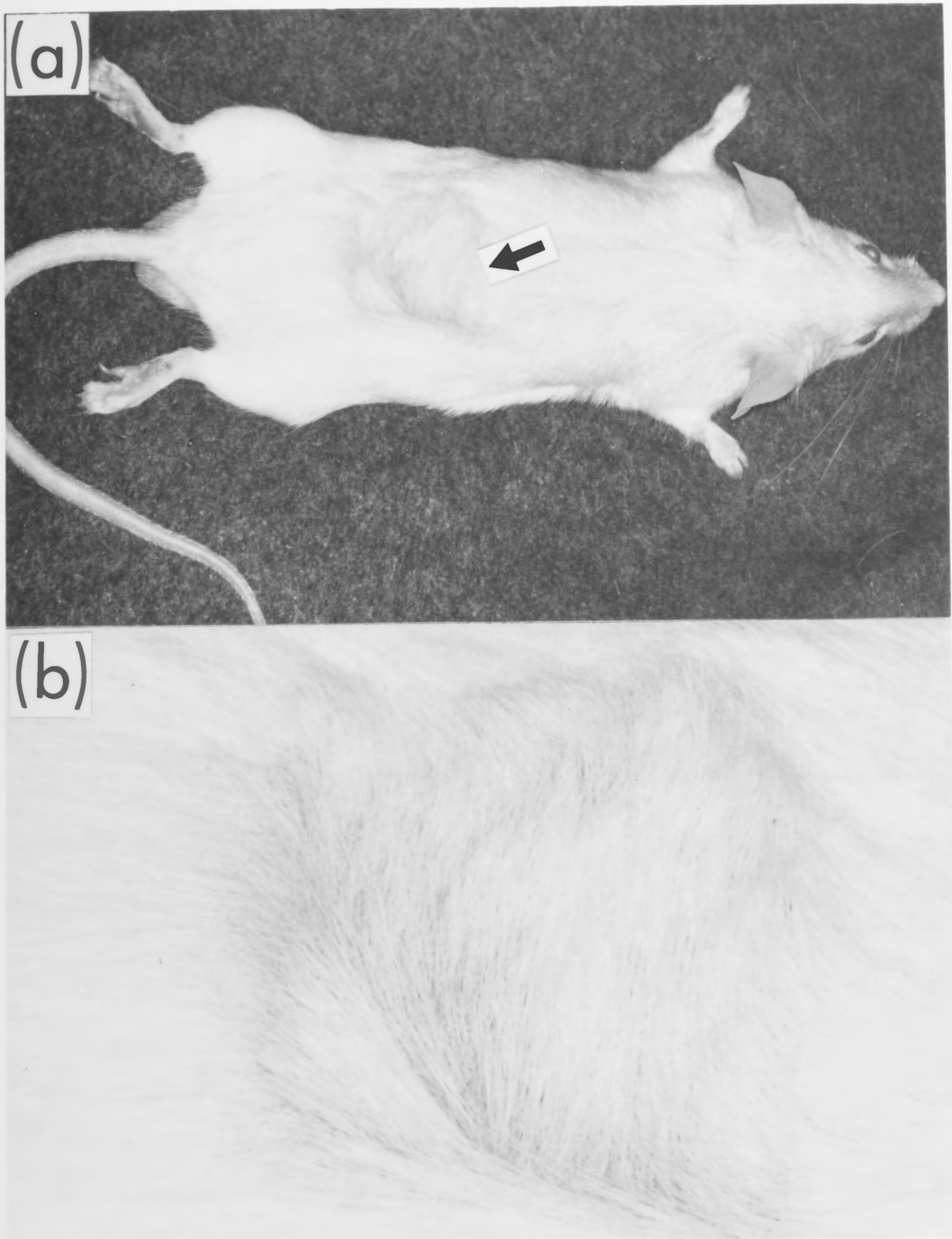


Figure 2.1 Macroscopic appearance of a BALB/c skin isograft at 435 days post-transplantation. (a) Note position of graft (indicated by arrow) on dorsal surface of body trunk. Magnification, x 1.1 . (b) Higher magnification of graft shows the counter direction of graft hair growth. Magnification, x 11 .

2.15 PREPARATION OF STREPTOZOTOCIN

2.15.1 Preparation of acetate buffer

A stock acetate buffer (pH 4.4) was prepared according to the following formula: 30.5 ml of 0.2M acetic acid, 19.5 ml of 0.2M sodium acetate, 0.9gm of sodium chloride. The solution was made up to a final volume of 100 ml with distilled water.

2.15.2 Drug formulation

Due to the instability of the drug following preparation (Rakieten *et al*, 1963), 30 mg of Streptozotocin (Calbiochem) were dissolved in 1 ml of acetate buffer (Section 2.15.1), immediately prior to use.

2.16 INDUCTION OF DIABETES AND MAINTENANCE OF DIABETIC MICE

Mice (9 - 12 weeks old) were made diabetic by a single tail vein injection of 300 mg/kg Streptozotocin (Section 2.15) on day 0. On days 3-6 (inclusive) post-Streptozotocin injection, the mice were given 1 ml of normal saline (Section 2.2) per gram of lost body weight, intraperitoneally, to prevent dehydration. In addition, the mice were maintained with a single daily subcutaneous injection of 0.8 units of Ultralente insulin (CSL) from day 3 following Streptozotocin injection until the time of transplantation. Diabetic mice were transplanted 10 to 41 days after Streptozotocin treatment. No insulin was administered on the day of transplantation. Insulin therapy was resumed the day following transplantation and maintained for 16-129 days.

2.17 BLOOD GLUCOSE MEASUREMENTS

Non-fasting blood glucose levels were assayed using either the Boehringer Mannheim GOD-Perid kit (Cat. No. 124 036) or a Beckman Glucose Analyser 2.

A 10 μ l blood sample was collected from the tail vein using a 10 μ l Microcap (Drummond, USA). The sample was transferred immediately to a microcentrifuge tube (Vetri; 1.5 ml with attached cap; Max Richter, West Germany) containing 40 μ l or 100 μ l of 0.66M perchloric acid for blood deproteinization, and vortexed to mix. For the GOD-perid glucose assay, 100 μ l of 0.66M perchloric acid was routinely used. Deproteinized samples were centrifuged for 2.5 minutes at approximately 8,740 g in a Beckman microcentrifuge. The supernatants were assayed for glucose content.

The GOD-perid kit measured glucose content on the basis of a colorimetric assay. Duplicate assays were set up for a glucose standard (0.51 mmoles/litre), test supernatants and a reagent blank. For each assay, an aliquot of 25 μ l was incubated with 1.25 ml of GOD enzyme reagent in a 37°C water bath for 15 minutes. The optical density (O.D.) of the glucose standard and test samples was then read against the reagent blank using a Unicam SP1800 spectrophotometer (Pye Unicam, England) set with a wavelength of 436 nm. The non-fasting blood glucose levels, corrected for sample dilution, were calculated according to the following formula:

$$\text{Non-fasting blood glucose} = 0.51 \times \frac{\text{sample dilution} \times \text{O.D. test average}}{\text{O.D. standard average}}$$

The lower limit of sensitivity for the assay was 0.16 mmoles

glucose/litre.

For determination of non-fasting blood glucose levels using the Beckman Glucose Analyzer 2, 10 μ l aliquots of test supernatants were assayed directly. Duplicate assays were averaged. Measurements were corrected for sample dilution and expressed in mmoles of glucose/litre. The lower limit of sensitivity for the analyser was 0.56 mmoles glucose/litre.

While mice were maintained daily on insulin, blood samples were taken for glucose measurement approximately 24 hours after the previous insulin dose. A time course study was carried out on blood glucose levels of diabetic mice following a subcutaneous injection of 0.8U Ultralente insulin (Fig. 2.2). Although there was a large variation in the response of different mice, any reduction in blood glucose levels was temporary and had dissipated by 24 hours post-injection.

Table 2.1 shows the mean non-fasting blood glucose levels of normal mice. Blood glucose levels above the upper 95% probability limit were considered hyperglycaemic. Figure 2.3 shows the blood glucose response of BALB/c mice following Streptozotocin injection. By 10 to 13 days post-Streptozotocin administration, the blood glucose levels were already hyperglycaemic and the mice showed a rapid loss in body weight. Thereafter, the blood glucose levels increased and remained in the upper reaches of the diabetic range for the duration of the study. While the mice were maintained daily on insulin, there was some recovery of lost body weight. However, following the termination of insulin therapy, the body weight level gradually declined.

Diabetic mice with blood glucose levels of more than 20.00 mmoles glucose/litre were generally selected for

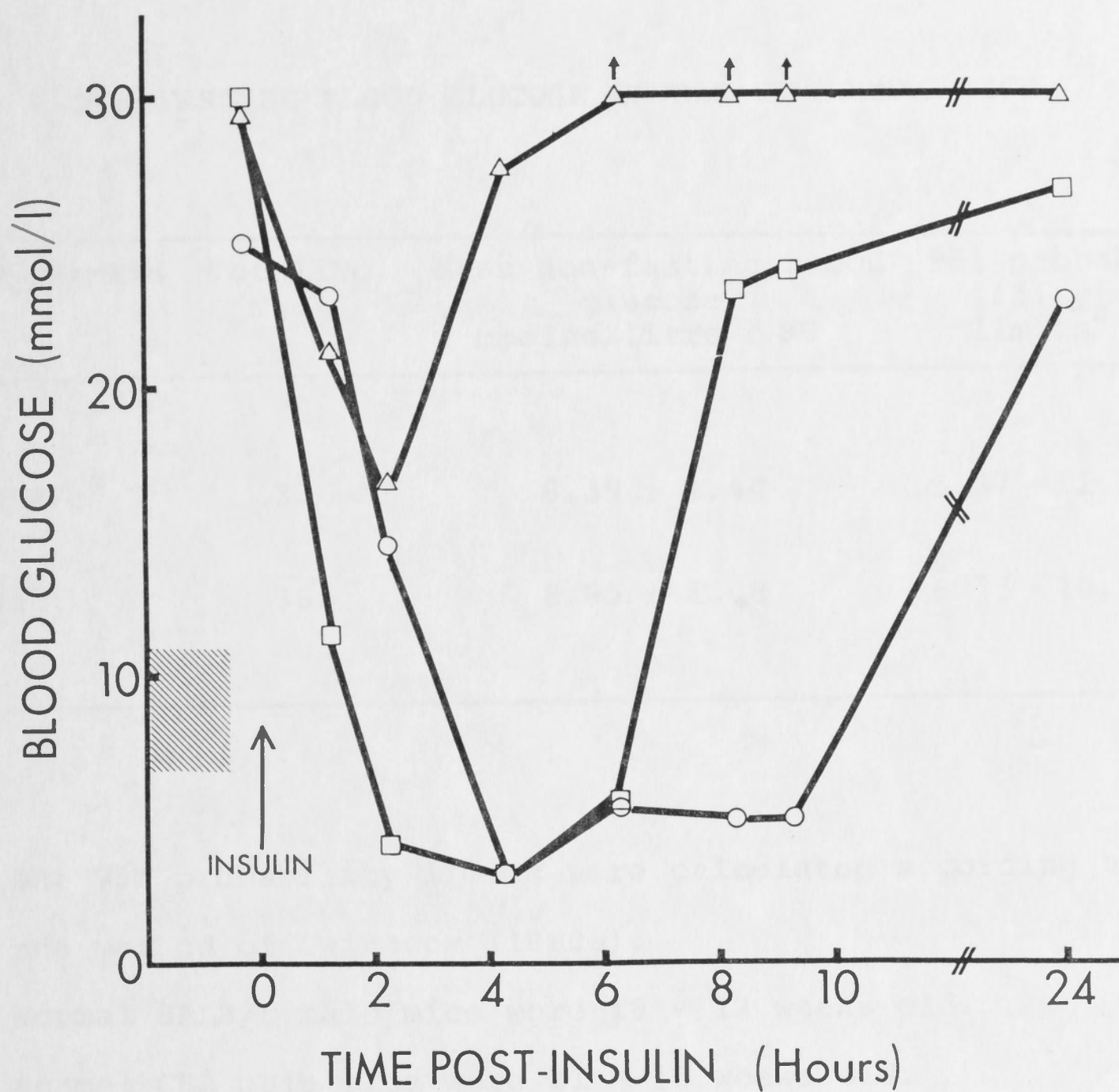


Figure 2.2 Non-fasting blood glucose response of diabetic CBA mice following subcutaneous administration of 0.8 units of Ultralente insulin. The data are representative of six animals. The shaded region defines the 95% probability interval for the non-fasting blood glucose levels of normal CBA mice.

TABLE 2.1

NON-FASTING BLOOD GLUCOSE LEVELS OF NORMAL MICE

Mouse strain	Total No. mice	Mean non-fasting blood glucose mmoles/litre \pm SD	95% probab- ility limits ^a
BALB/c ^b	33	8.39 \pm 1.44	5.57 - 11.21
CBA ^c	36	8.85 \pm 1.08	6.73 - 10.97

- a. The 95% probability limits were calculated according to the method of Swinscow (1980a).
- b. Normal BALB/c male mice were 10 - 12 weeks old.
- c. Normal CBA male mice were 11 - 13 weeks old.

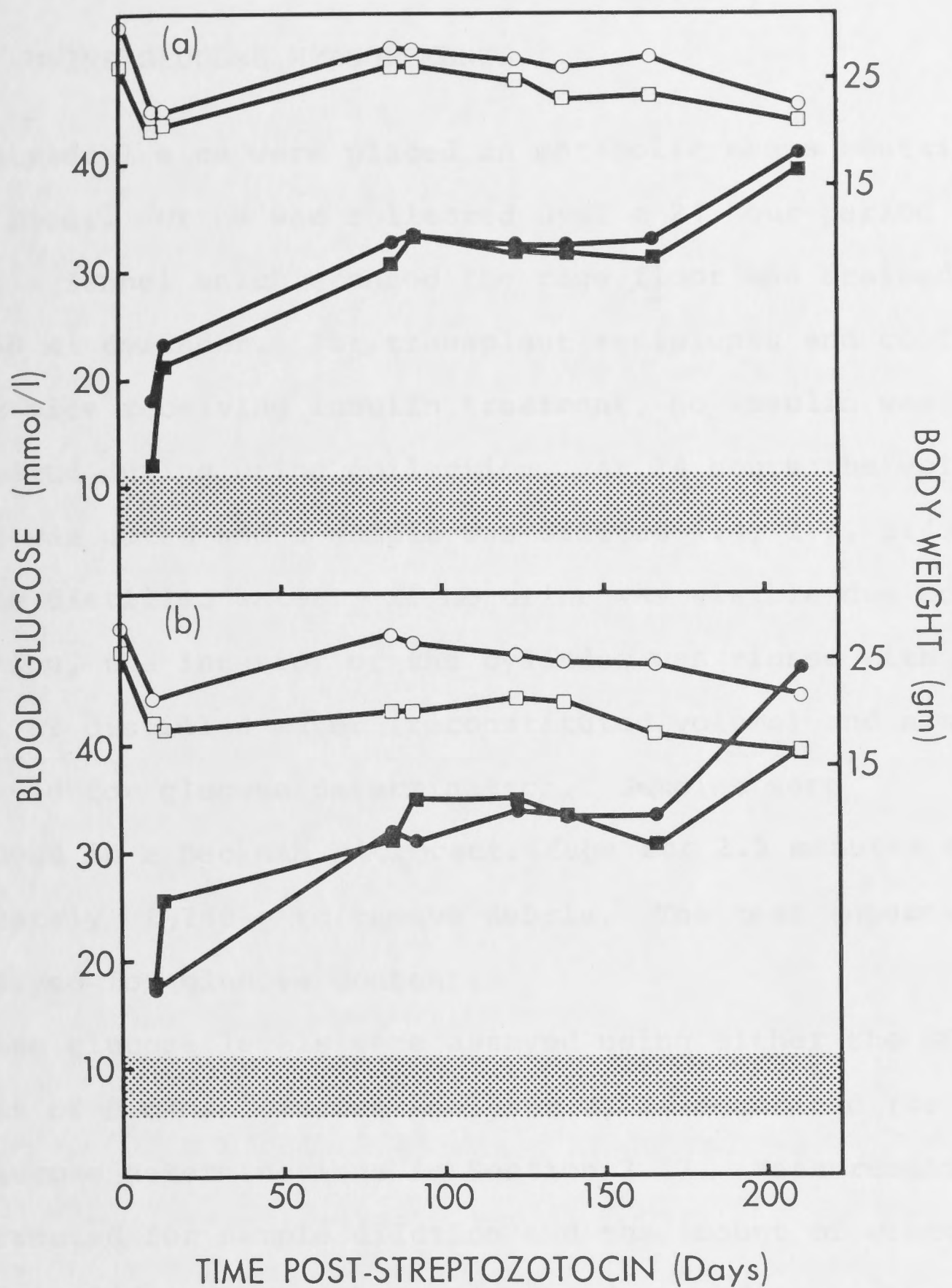


Figure 2.3 Non-fasting blood glucose levels (closed symbols) and body weight (open symbols) of BALB/c male mice following intravenous administration of Streptozotocin (300 mg/kg) on day 0. The mice were maintained with 0.8U Ultralente insulin/day on days 3-131 (inclusive) and then 0.4U insulin for the following two days. Insulin treatment was then terminated. Each panel shows the data for two mice. The shaded region represents the 95% probability interval for the non-fasting blood glucose levels of normal BALB/c mice. The data are representative of a total group of five Streptozotocin-treated mice.

transplantation.

2.18 URINE GLUCOSE MEASUREMENTS

Individual mice were placed in metabolic cages containing a gauze floor. Urine was collected over a 24 hour period by means of a funnel which spanned the cage floor and drained into a 50 ml cylinder. For transplant recipients and control diabetic mice receiving insulin treatment, no insulin was administered during urine collection. At 24 hours the volume of urine was noted and a sample was diluted 1:4, 1:9, 1:49 or 1:99 with distilled water. If no urine was visible due to evaporation, the interior of the cylinder was rinsed with 1 - 2 ml of distilled water (reconstituted volume) and a sample was removed for glucose determination. Samples were centrifuged in a Beckman microcentrifuge for 2.5 minutes at approximately 8,740 g to remove debris. The test supernatants were assayed for glucose content.

Urine glucose levels were assayed using either the GOD-perid kit or Beckman Glucose Analyser 2, as described for blood glucose determinations in Section 2.17. Measurements were corrected for sample dilution and the amount of glucose excreted in 24 hours was then calculated according to the following formula:

$$\begin{array}{lcl} \text{Urine glucose} & & 24 \text{ hr urine volume} \\ \text{output} & = & \text{or} \\ (\text{mmoles}/24 \text{ hours}) & & \text{reconstituted volume} \end{array} \times \frac{\text{mmoles glucose/litre}}{1000}$$

(ml)

Urine glucose levels <0.005 mmoles glucose/24 hours did not fall within the range of accuracy for these assays.

Urine glucose measurements of normal BALB/c and CBA mice are presented in Table 2.2 and Table 2.3 respectively. Normal mice may excrete small quantities of glucose in their urine. Alternatively, the low glucose levels may be due to glucose extraction from food grains which accumulate in the urine collection cylinders. It is likely, therefore, that the procedure used for measuring urine glucose levels does not yield accurate determinations of the amount of glucose actually excreted in urine. However, the urine glucose levels of diabetic mice were approximately 2-3 orders of magnitude higher than for normal mice. On this basis, urine glucose measurements were used to monitor the function of foetal pancreas grafts.

2.19 SURGICAL REMOVAL OF GRAFT-BEARING KIDNEY

Transplant recipients were anaesthetized with avertin anaesthetic (Section 2.12). A vertical incision was made in the left flank, next to the incision site used for transplantation (see Section 2.13). The graft-bearing kidney was exposed so that the main renal artery and vein were visible. These blood vessels were ligated with sterile, atraumatic surgical silk (Size 000; Ethicon, USA). The kidney was cut free and the excision site was swabbed with HEPES-buffered HBSS (Section 2.2). The incised skin was sutured together with autoclips (9mm; Clay Adams, USA). Non-fasting blood glucose levels of mice were measured regularly during the first week after nephrectomy and at random intervals thereafter.

Figure 2.4 shows the non-fasting blood glucose levels and body weight of normal, non-transplanted BALB/c mice

TABLE 2.2

URINE GLUCOSE OUTPUT

Mouse strain	Treatment	Total No. mice	Urine glucose (mmoles/24 hr)	
			Day A	Day B
BALB/c	Normal ^a	7	0.00	0.00
			0.00	0.00
			0.00	0.00
			0.00	0.00
			0.00	0.00
			0.00	0.00
			0.00	0.00
BALB/c	Diabetic ^b	5	11.93	-
			9.38	-
			10.11	-
			10.84	-
			10.21	-

a. Normal mice were approximately 12.5 weeks and 22 weeks old on Days A and B respectively.

b. Diabetic mice were tested at 181-186 days post-Streptozotocin injection.

TABLE 2.3

URINE GLUCOSE OUTPUT

Mouse strain	Treatment	Total No. mice	Urine glucose (mmoles/24 hr)	
			Day A	Day B
CBA	Normal ^a	12	0.01	0.00
			0.01	0.00
			0.01	0.00
			0.01	0.00
			0.01	0.00
			0.02	0.00
			0.01	0.00
			0.01	0.00
			0.01	0.00
			0.01	0.00
			0.04	0.00
			0.02	0.00
CBA	Diabetic ^b	10	5.22	4.7
			0.99	17.40
			4.56	8.50
			3.60	17.25
			6.38	14.72
			8.04	8.40
			8.73	12.06
			8.43	9.95
			23.40	9.36
			2.42	18.36

a. Normal mice were approximately 14 - 15 weeks and 24 - 25 weeks old on Days A and B respectively.

b. Diabetic mice were 10 - 22 days and 49 - 51 days post-Streptozotocin injection on Days A and B respectively.

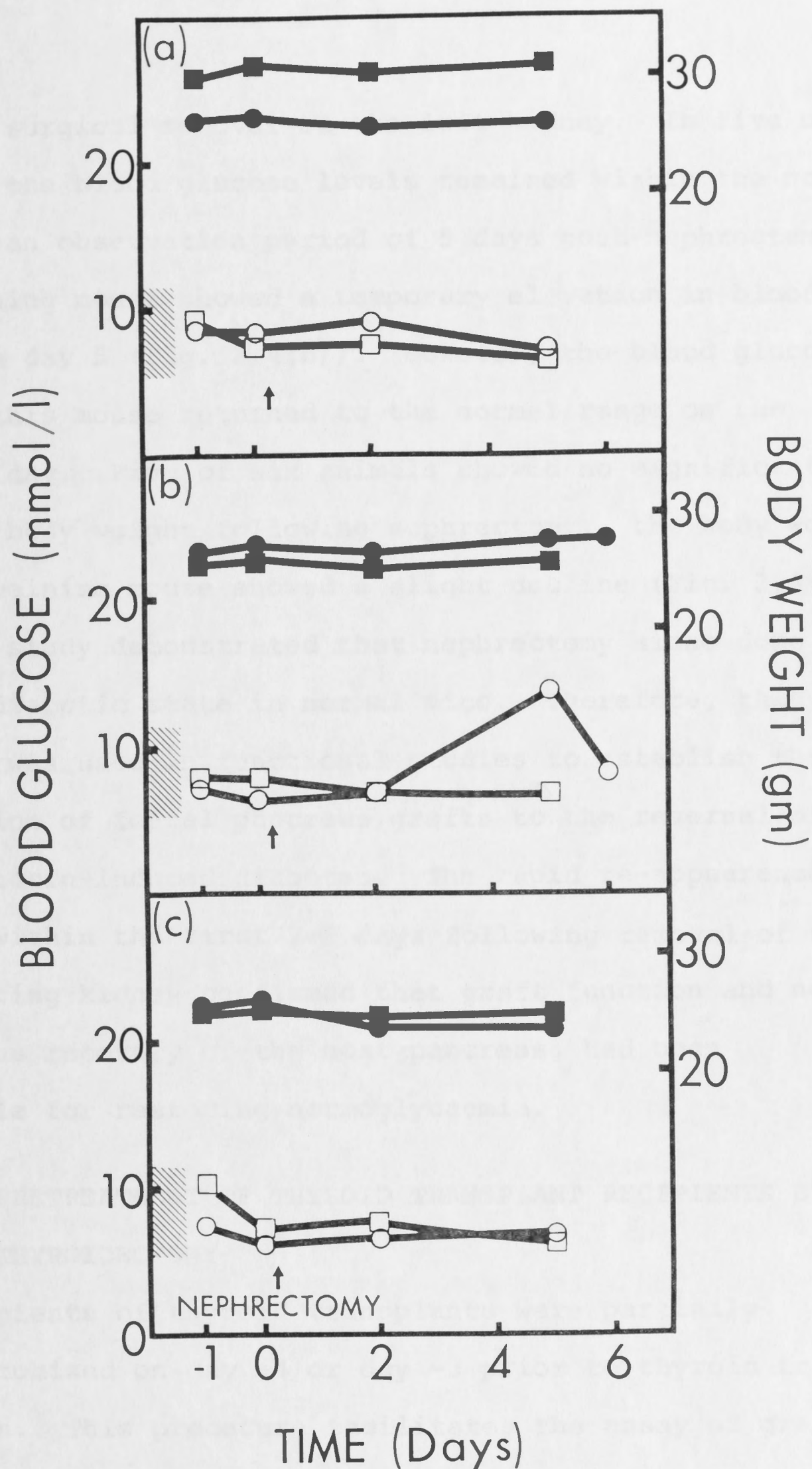


Figure 2.4 The non-fasting blood glucose levels (open symbols) and body weight (closed symbols) of normal BALB/c mice following nephrectomy of the left kidney. Each panel shows the data for 2 animals. The shaded region defines the 95% probability interval for the non-fasting blood glucose levels of normal BALB/c mice.

following surgical removal of the left kidney. In five of six mice, the blood glucose levels remained within the normal range for an observation period of 5 days post-nephrectomy. The remaining mouse showed a temporary elevation in blood glucose on day 5 (Fig. 2.4(b)). However, the blood glucose level of this mouse returned to the normal range on the following day. Five of six animals showed no significant change in body weight following nephrectomy; the body weight of the remaining mouse showed a slight decline (Fig. 2.4(c)).

This study demonstrated that nephrectomy alone does not induce a diabetic state in normal mice. Therefore, this procedure was used in functional studies to establish the contribution of foetal pancreas grafts to the reversal of Streptozotocin-induced diabetes. The rapid re-appearance of diabetes within the first 2-5 days following removal of the graft-bearing kidney confirmed that graft function and not spontaneous recovery of the host pancreas, had been responsible for restoring normoglycaemia.

2.20 PRETREATMENT OF THYROID TRANSPLANT RECIPIENTS BY THYROIDECTOMY

Recipients of thyroid transplants were partially thyroidectomized on day -4 or day -3 prior to thyroid transplantation. This procedure facilitates the assay of graft function (Section 2.21) and stimulates the production of thyroid-stimulating hormone, thereby promoting the growth of grafted thyroid tissue (Lafferty *et al.*, 1975).

The mice were anaesthetized with avertin (Section 2.12) and the hair over the throat was removed by scraping with a large scalpel blade (Size 22; Swann-Morton, England). The

site was swabbed with 70% ethanol and a vertical mid-line incision was made in the skin. The underlying salivary glands were teased apart and a vertical incision was made in the muscle layers over the trachea. The muscle was parted to expose the two thyroid lobes, one lobe on either side of the trachea. As much as possible of each thyroid lobe was then excised by blunt dissection. Care was taken to avoid damage to the recurrent laryngeal nerve which runs close to the left thyroid lobe. The muscle layer was replaced over the trachea and the incised skin was sutured with autoclips (9mm; Clay Adams, USA). The mice were then placed under an incandescent lamp until they recovered from the anaesthesia.

2.21 MEASUREMENT OF THYROID FUNCTION

Thyroid function was assessed according to the procedure described by Lafferty *et al* (1976a). In brief, transplant recipients received an intraperitoneal injection of 15 μ Ci 125 Iodine (125 I; IMS.30, Amersham, England) at 3 weeks post-transplantation and at 3 week intervals thereafter. Three days after 125 I administration, the mice were anaesthetized with avertin (Section 2.12) and the level of radiation emitted from the thyroid transplant was measured by positioning a scintillation probe above the site of the grafted kidney (Lafferty *et al*, 1976a).

The criterion for absence of graft function was arbitrarily chosen as three standard deviations above the mean uptake of 125 I (c.p.m.) by uncultured BALB/c (H-2^d) thyroid allografts at 24 days after transplantation to thyroidectomized CBA(H-2^k) recipient mice (Section 2.20). Based on a group of 6 transplanted mice, this value was 873 c.p.m.. Grafts

measuring ≤ 873 c.p.m. were re-tested the following week for confirmation. Mice carrying grafts which measured ≤ 873 c.p.m. on two consecutive weeks were sacrificed and the thyroid grafts were taken for histological examination (Section 2.26).

2.22 PREPARATION OF PERITONEAL CELLS

Each donor mouse was killed by cervical dislocation, and the abdomen was swabbed with 70% ethanol. A small incision was made in the abdominal skin and the skin was then gently peeled back to expose the peritoneum. The peritoneal cavity was irrigated with 2.5 ml of HEPES-buffered HBSS (Section 2.2) supplemented with heparin (20 U/ml). The cell suspension was withdrawn into a syringe, transferred to a 10 ml centrifuge tube and centrifuged at 800 g for 5 minutes. The cell pellet was resuspended in 1 ml of heparin-free HEPES-buffered HBSS. Cell viability was determined using the trypan blue exclusion test and the density of viable cells was assessed using a haemocytometer (Bright-Line, USA).

2.23 PREPARATION OF MOUSE THYMOCYTES

Thymus lobes were removed from 6 young BALB/c mice (5-7 weeks old) and minced with scissors on a fine stainless steel grid. To prepare the cell suspension, the minced tissue was pressed through the grid into 4-5 ml of HBSS (Section 2.2), buffered with 19.6mg sodium bicarbonate/100 ml. The thymocyte suspension was transferred to a graduated centrifuge tube and a sample was removed for determination of cell density using a haemocytometer (Bright-Line, USA). The cell density was 8.4×10^7 thymocytes/ml.

2.24 PREPARATION OF RABBIT ANTI-MOUSE LYMPHOCYTE SERUM (ALS)

2.24.1 Immunization

Equal volumes of a suspension of mouse thymocytes (Section 2.23) and Complete Freund's Adjuvant (Difco Laboratories, USA) were mixed to produce an emulsion for immunization. The protocol for immunization was based on the method described by Parish *et al* (1976). Two young adult rabbits were immunized on day 0 with a subcutaneous injection of 6.3×10^7 thymocytes in a total volume of 1.5 ml of emulsion. On day 14 the same rabbits received an intravenous booster injection of 6.0×10^7 thymocytes prepared in 1 ml of sodium bicarbonate-buffered HBSS (Section 2.23).

2.24.2 Serum collection

On day 21 a blood sample was withdrawn from the ear vein of both rabbits. On day 25, the rabbits were anaesthetized with an intravenous injection of 1 ml of Nembutal (sodium pentobarbitone, 60 mg/ml; Abbott Laboratories, Australia) and bled out via a cardiac puncture. On both occasions the collected blood was placed, for 2 hours, in a constant temperature room maintained at 37°C , to promote clot formation. The clotted blood was stored at 10°C overnight to facilitate clot contraction. On the following day, the crude serum was collected and centrifuged at 1380 g for 10 minutes, to remove debris. The batches of serum prepared from the same rabbit were pooled; the sera prepared from different rabbits were stored separately, in aliquots, at -20°C .

2.24.3 Absorption

Before use, the ALS was absorbed with mouse red blood cells to remove anti-erythrocyte reactivity.

BALB/c mice were anaesthetized with ether (Hoechst, Australia) and bled out from the major vessels of the axilla. The blood was collected in Alsevers solution (Section 2.2), held on ice, and centrifuged at 450 g for 7 minutes. The supernatant was removed and the red blood cells were transferred to a graduated centrifuge tube, washed in PBS (Section 2.2) and centrifuged at 635 g for 5 minutes. The supernatant was removed and the volume of packed red blood cells was noted. The ALS was absorbed by suspending 1 part of packed red blood cells in 2 parts of ALS, at 4°C for 2 hours. The suspension was then centrifuged at 635 g for 5 minutes. The absorbed serum was removed and stored in aliquots, at -20°C.

The absorbed preparations of ALS had cytotoxic antibody titres of $\geq 1:256$ on BALB/c peritoneal cells, using trypan blue exclusion as an indicator of cell viability (Carlson and Terres, 1976).

2.25 TREATMENT OF TRANSPLANT RECIPIENTS WITH ALS

Antilymphocyte serum (ALS) was prepared according to the procedure described in Section 2.24.

For ALS treatment, mice received an intravenous injection of 0.25 ml of absorbed ALS (Section 2.24.3) approximately 45-90 minutes prior to transplantation, followed by 0.25 ml of absorbed ALS intraperitoneally on days 1, 3 and 5 post-transplantation.

2.26 HISTOLOGY

2.26.1 Preparation of formol saline

Formol saline was prepared according to the following formula: 100 ml of formaldehyde; sodium chloride, 8.5gm; monosodium hydrogen phosphate, 4.0gm; disodium hydrogen phosphate, 6.5gm. The solution was made up to 1 litre with distilled water.

2.26.2 Preparation of tissue for histological examination

For histological studies of tissue transplants, transplant recipients were sacrificed at appropriate intervals after transplantation. The graft-bearing kidney was removed and the graft was fixed *in situ* with formol saline (Section 2.26.1). Serial sections at 4 or 100 μ intervals were stained with both haematoxylin with eosin and aldehyde fuchsin. Aldehyde fuchsin specifically stains the beta (β) cells of pancreatic islets (Bussolati and Bassa, 1974). Sections of thyroid and skin grafts were stained only with haematoxylin with eosin.

To facilitate histological processing of cultured foetal pancreas, digested foetal pancreas or isolated proislets, the tissue samples were fixed with approximately 1 ml of formol saline in microcentrifuge tubes (Vetri; 1.5 ml with attached cap; Max Richter, West Germany) for > 2 days. The fixative was then removed and replaced with approximately 1 ml of melted 3% agar. The samples were immediately centrifuged for 2.5 minutes at approximately 8,740 g in a Beckman micro-centrifuge. The 3% agar was melted in a boiling water bath and allowed to cool, but not solidify, prior to use.

CHAPTER 3

ORGAN CULTURE AND ISOTRANSPLANTATION OF

FOETAL MOUSE PANCREAS

3.1 INTRODUCTION

Foetal pancreas and isolated adult islets represent the main sources of donor tissue for experimental pancreatic islet transplantation. However, the foetal organ offers several advantages over the use of adult islet tissue. Following isotransplantation of foetal pancreas, the exocrine component degenerates without activation of exocrine enzymes and autodigestion of endocrine tissue (Coupland, 1960; Brown *et al.*, 1974, 1976; Hegre *et al.*, 1976a). Hence, in contrast to adult pancreas tissue, the separation and removal of exocrine tissue before transplantation is unnecessary. A further distinctive property of the foetal tissue is the potential for further growth of the endocrine component following transplantation (Browning and Resnik, 1951-1952; Hegre *et al.*, 1976a; McEvoy and Hegre, 1978, 1979). Using the morphometric technique of linear scanning, McEvoy and Hegre (1978) examined isografts composed of eight foetal rat pancreases at 15 days after transplantation under the kidney capsule of alloxan-treated diabetic recipients. They calculated that an eight-fold increase in beta cell mass and a five-fold increase in insulin content per beta cell occurred during the transplant period. An absolute requirement for the practical application of foetal pancreas for transplantation is the provision of islet function. Evidence that transplanted foetal pancreas tissue retains the capacity to differentiate and to develop endocrine function has been provided by reports that isografts of multiple foetal rat pancreases can reverse experimentally-induced diabetes in recipient animals

(Brown *et al.*, 1974, 1976; Hegre *et al.*, 1976a; Spence *et al.*, 1977; McEvoy *et al.*, 1978; McEvoy and Hegre, 1979).

The major obstacle to the transplantation of foetal pancreas for the reversal of experimentally-induced diabetes lies in the immunological problem of allograft rejection (Browning and Resnik, 1951-1952; Garvey *et al.*, 1979a). As mentioned previously (Section 1.6.3), organ culture in a high oxygen gas phase has been shown to facilitate the allotransplantation (Lafferty *et al.*, 1975, 1976a) and xenotransplantation (Sollinger *et al.*, 1977) of thyroid tissue in rodents. Furthermore, support for a direct role of oxygen in modulating tissue immunogenicity was provided by the finding that the culture duration required to obtain survival of mouse thyroid allografts could be substantially reduced by increasing the partial pressure of oxygen in the gas phase (Talmage and Dart, 1978). On the basis of this evidence, our first objective was to examine the capacity of the organ culture technique to reduce the immunogenicity of foetal mouse pancreas allografts. In preparation for this study (detailed in Chapter 4), initially we investigated the technical aspects of maintaining foetal mouse pancreas in culture under a 95% O₂, 5% CO₂ gas phase. The organ culture procedure has been used previously by other groups to investigate the cellular differentiation of foetal pancreas tissue and the development of endocrine function under strict environmental conditions. In this context, it was reported that foetal rat pancreas can survive organ culture for 8-12 days in a high oxygen atmosphere (Schweisthal *et al.*, 1965; Murrell, 1966; Wells *et al.*, 1967; Hegre *et al.*, 1972). In contrast

to these findings, Chase and co-workers (1979) found that a high oxygen gas phase was toxic to foetal mouse pancreas if the tissue was cultured for longer than 4 days. Thus discrepancies exist in the literature concerning the ability of foetal pancreas tissue to tolerate high oxygen levels *in vitro*.

In this chapter we examine the differentiation and functional development of foetal mouse pancreas following isotransplantation at the renal subcapsular site. These data establish baseline properties of the untreated foetal tissue. We investigate the survival of foetal mouse pancreas following organ culture in a 95% O₂, 5% CO₂ gas phase and isotransplantation. These findings are used to establish optimal culture conditions for an investigation of the effect of organ culture on foetal pancreas immunogenicity (see Chapter 4). Finally, we document a study of the functional integrity of cultured foetal pancreas tissue following isotransplantation.

3.2 RESULTS

3.2.1 Histological description of foetal mouse pancreas

At 17 days of gestation, foetal mouse pancreas explants consisted predominantly of acinar tissue at various stages of differentiation, undifferentiated tissue and some duct-like epithelium. The majority of undifferentiated tissue resembled islet tissue (Fig. 3.1(a)). Unlike mature islets, the regions of undifferentiated tissue were generally pleiomorphic, and were neither characterized by a well defined boundary nor stained with aldehyde fuchsin.

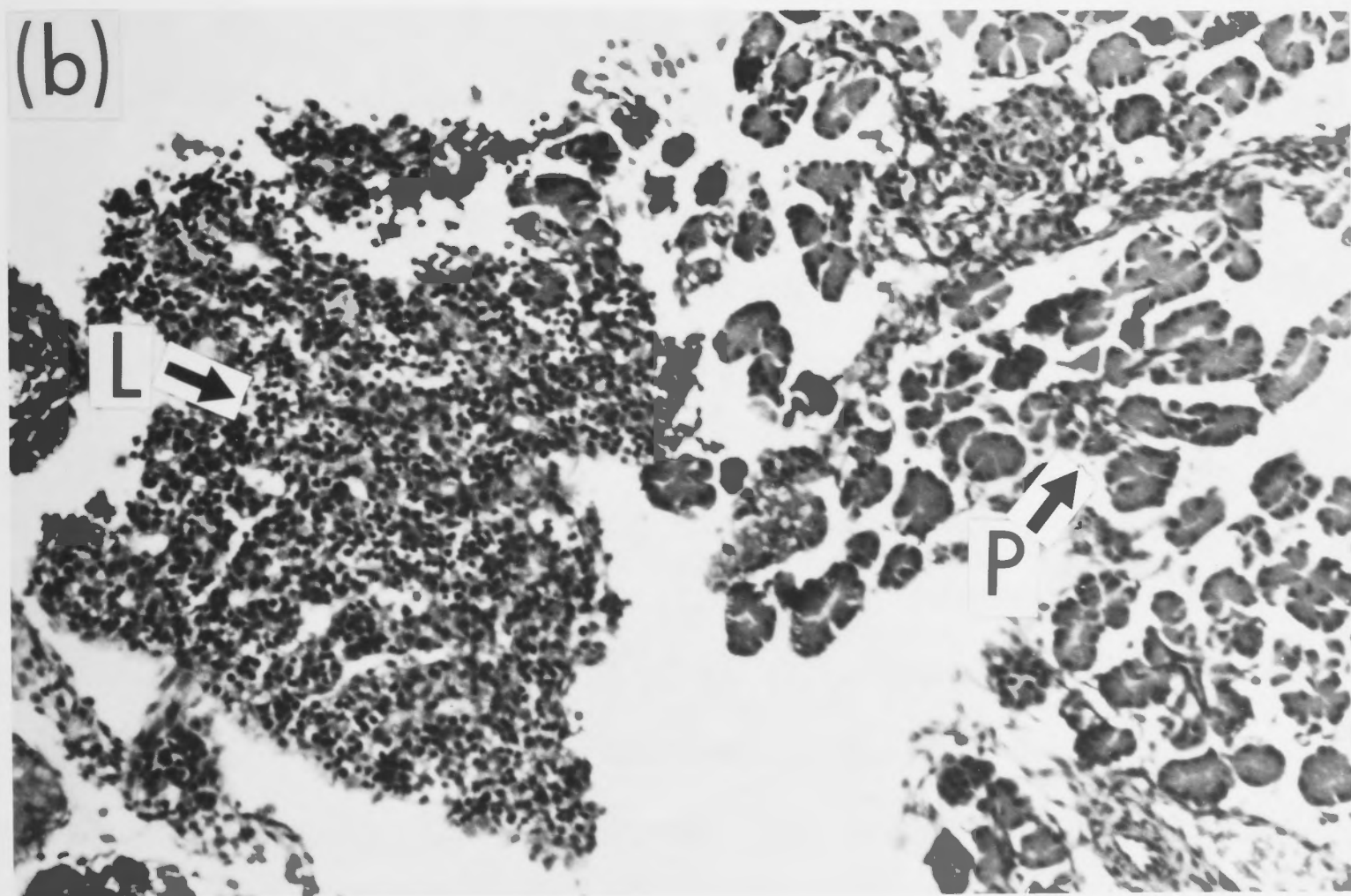
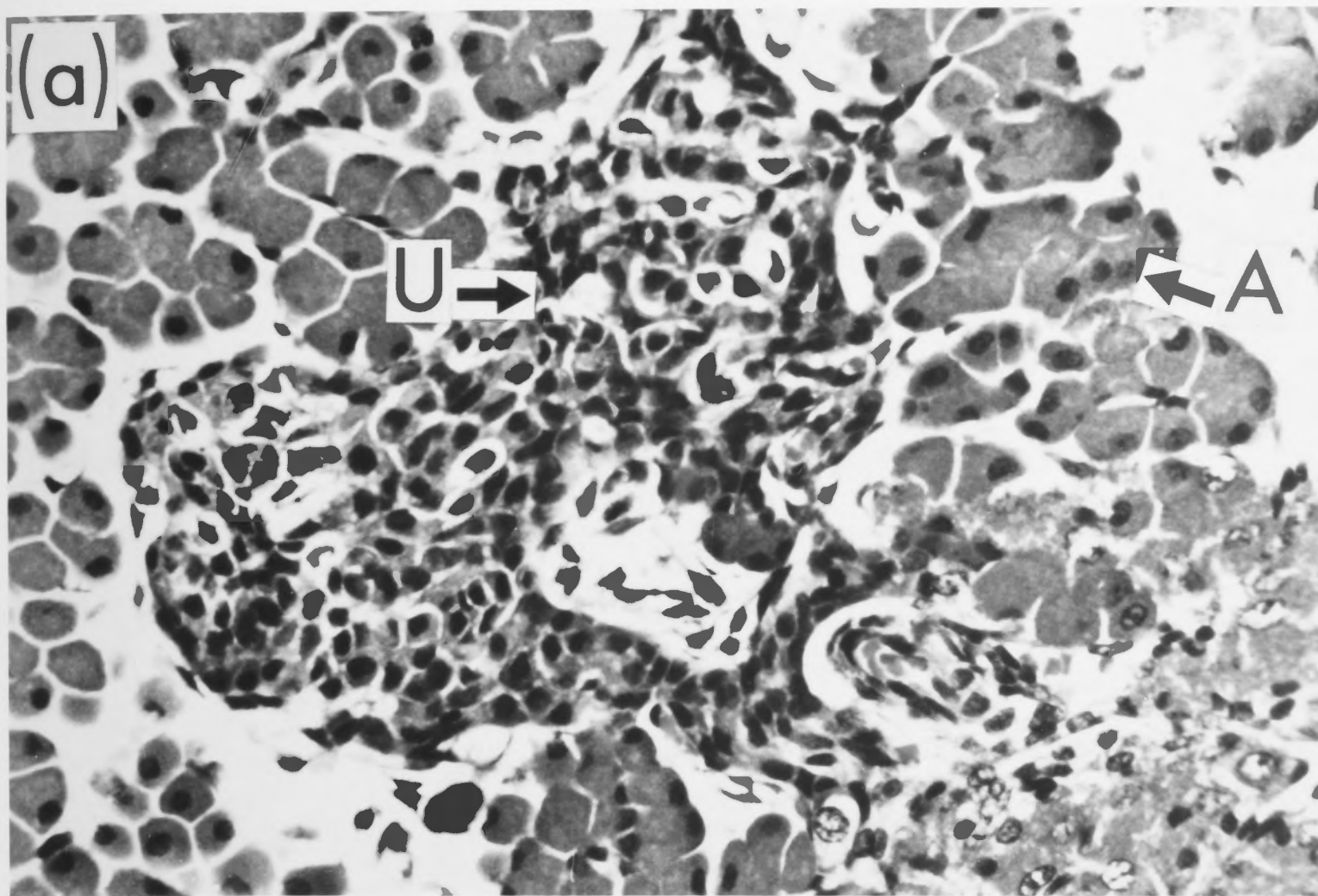


Figure 3.1 Histological appearance of BALB/c foetal mouse pancreas at 17 days of gestation. Panel (a) shows undifferentiated tissue (U) resembling islet tissue, and surrounding acinar tissue (A). Haematoxylin and eosin, x 423. Panel (b) shows primitive lymphoid tissue (L) adjacent to pancreatic tissue (P). Haematoxylin and eosin, x 174.

In addition, primitive lymphoid tissue was found closely associated with the periphery of foetal pancreas explants (Fig. 3.1(b)). This lymphoid component was characterized by loosely packed, small, round cells which resembled mononuclear leukocytes. Table 3.1 shows that associated lymphoid tissue was identified in four of six foetal pancreases which were examined. In two of the four positive samples, serial histological sections at 4μ intervals were examined; in the case of the two negative samples the tissue was sectioned at 100μ intervals. This finding suggests that routine identification of the primitive lymphoid component of foetal pancreas probably requires thorough histological examination of the entire organ.

3.2.2 Isotransplantation of foetal mouse pancreas

We first set out to investigate the ability of foetal pancreas to survive and differentiate following isotransplantation under the kidney capsule of BALB/c recipient mice. At 2 to 4 weeks after transplantation the isografts were revascularized and contained well differentiated, aldehyde fuchsin-positive islets and some duct epithelium (Fig. 3.2). By 4 weeks post-transplantation there was a massive accumulation of adipose tissue within the grafts. The majority of exocrine tissue had degenerated and only small patches of acini remained. Often islets and small acini were found closely associated with duct epithelium. In addition, a proportion of isografts contained a large mass of lymphoid tissue which structurally resembled a lymph node (Fig. 3.3).

TABLE 3.1

Lymphoid tissue associated with uncultured foetal mouse
pancreas before and after isotransplantation

Tissue	Total No. examined	No. with assoc- iated lymphoid tissue	% samples showing lymphoid contamination
Foetal pancreas	6 ^a	4 ^b	67
Foetal pancreas isograft ^c	12 ^d	7 ^e	58

- a.* Two of six pancreases were serially sectioned at 4 μ intervals; remaining pancreases were sectioned at 100 μ intervals.
- b.* Two of four pancreases were serially sectioned at 4 μ intervals.
- c.* Isografts were examined histologically at 2 - 4 weeks after transplantation.
- d.* Four of twelve isografts were serially sectioned at 4 μ intervals; remaining isografts were sectioned at 100 μ intervals.
- e.* Three of seven isografts were serially sectioned at 4 μ intervals.

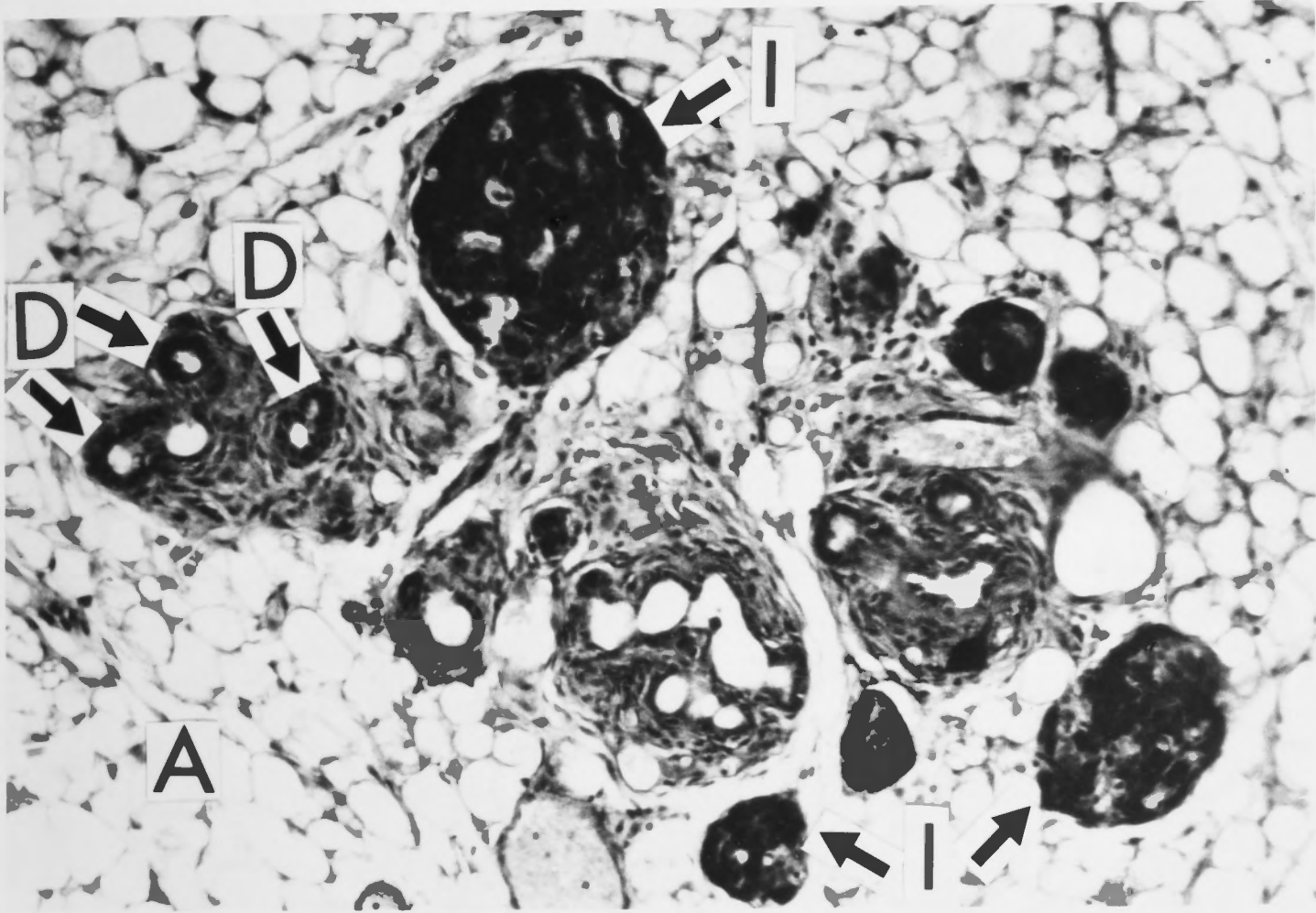


Figure 3.2 Isograft of BALB/c foetal mouse pancreas 4 weeks after transplantation under the kidney capsule. Note aldehyde fuchsin-positive islets (I), duct epithelium (D) and massive amount of adipose tissue (A). Aldehyde fuchsin, x 171.

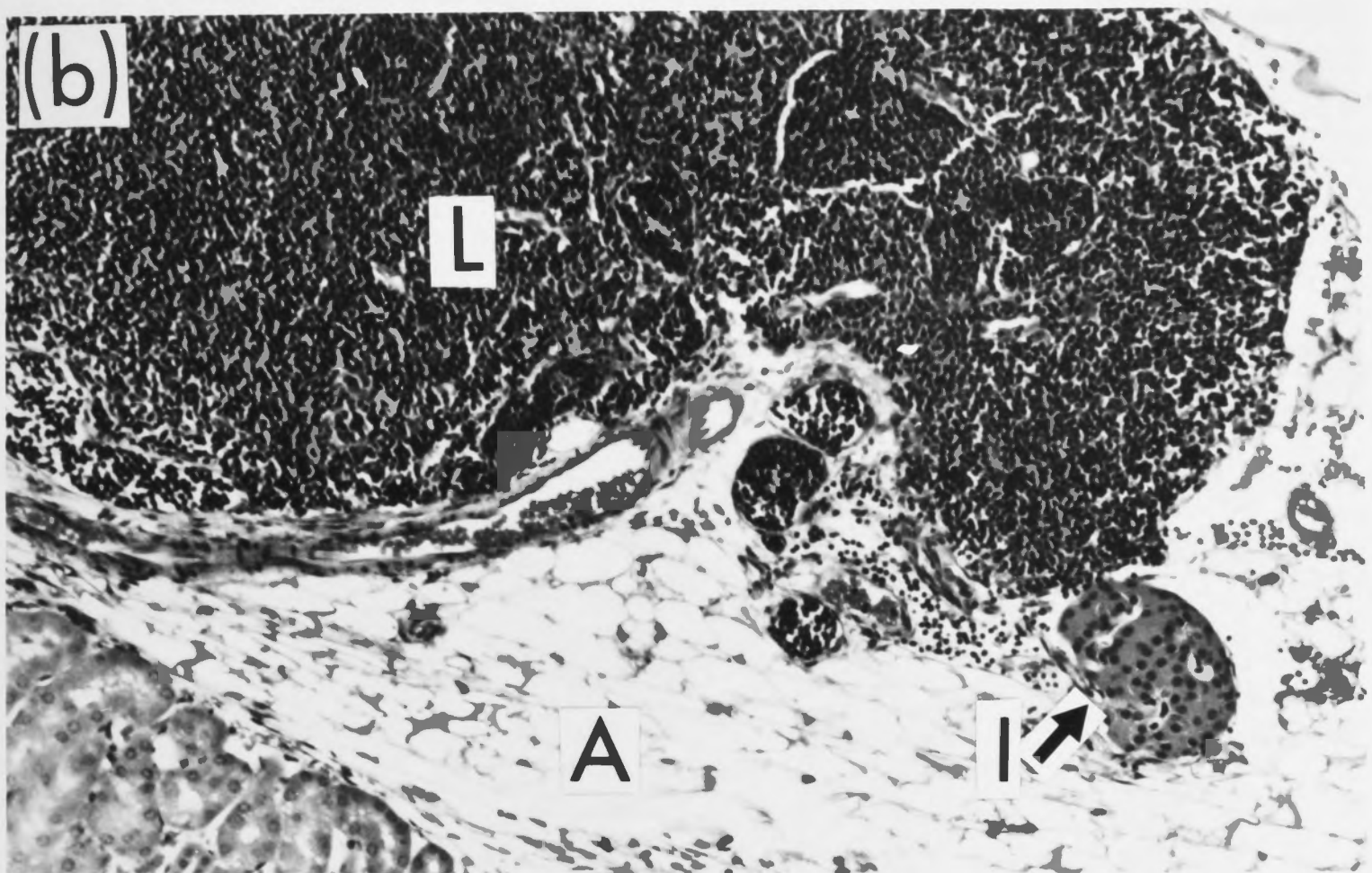
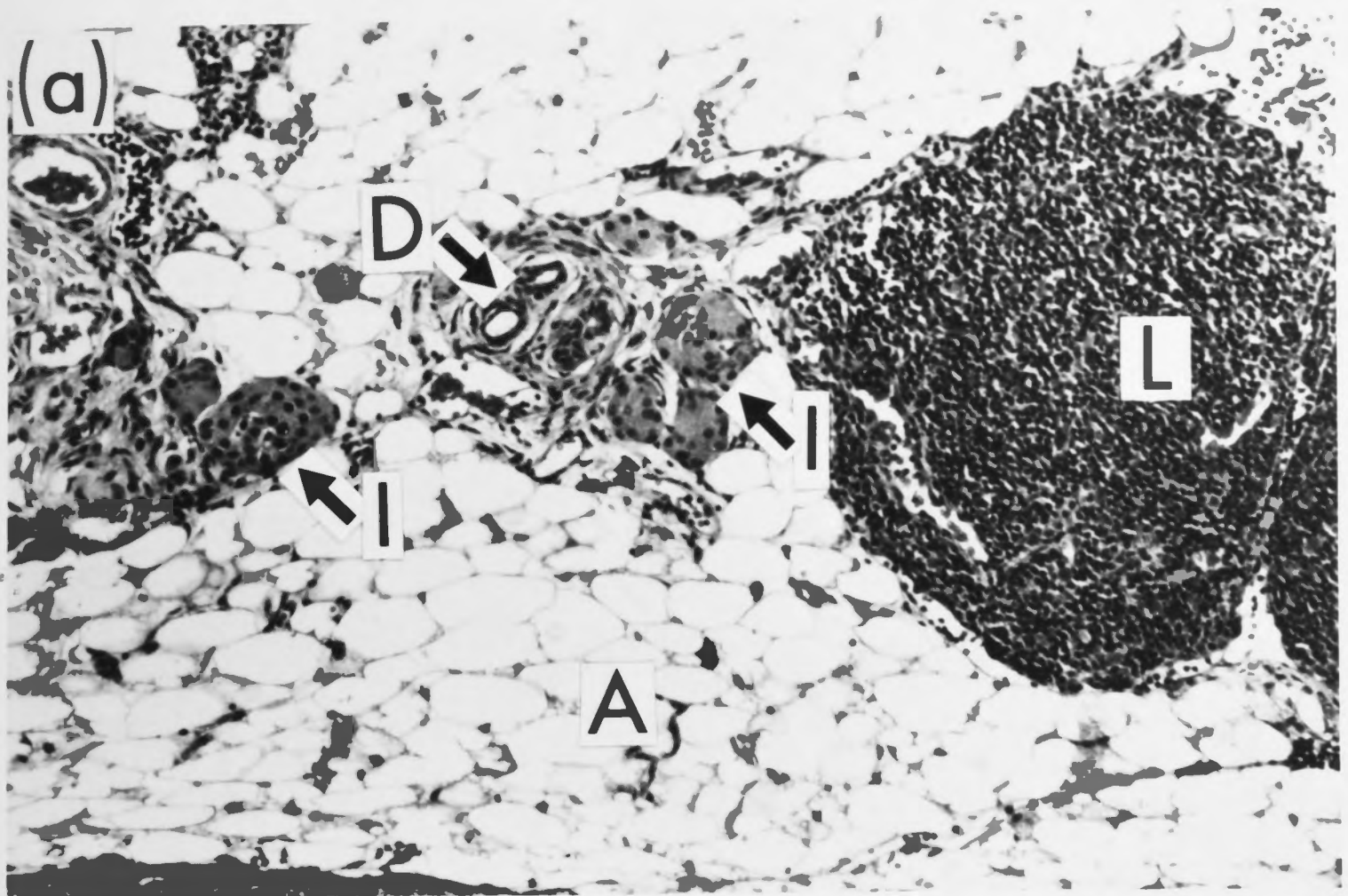


Figure 3.3 Panels (a) and (b) each show an isograft of BALB/c foetal mouse pancreas 4 weeks after transplantation beneath the kidney capsule. Note presence of islets (I), duct tissue (D), adipose tissue (A) and mass of lymphoid tissue (L). (a) Haematoxylin and eosin, x 167. (b) Haematoxylin and eosin, x 173.

Table 3.1 shows that seven of twelve isografts examined at 2 to 4 weeks after transplantation contained lymph-node-like structures. Examination of four grafts serially sectioned at 4μ intervals showed that three of the four grafts contained a mass of lymphoid tissue; of eight grafts sectioned at 100μ intervals, four grafts contained lymph-node-like structures. These findings support the detection of primitive lymphoid tissue associated with foetal pancreas explants (Section 3.2.1). Thus, it appears that the primitive lymphoid component is co-transplanted with the foetal pancreas and, following transplantation, continues to develop into a more organized lymphoid structure. As in the case of foetal pancreas explants, detection of the associated lymphoid tissue of foetal pancreas isografts may be facilitated by the histological examination of serial sections through the entire graft.

In addition, the histological data confirm the findings of other groups (Browning and Resnik, 1951-1952; Brown *et al.*, 1976; Hegre *et al.*, 1976a) that following isograft transplantation of foetal pancreas, the exocrine component degenerates and the endocrine component continues to grow and to yield differentiated islets.

3.2.3 Function of foetal pancreas isografts

The minimum number of foetal mouse pancreases required to reverse Streptozotocin-induced diabetes was examined. Figure 3.4 shows the results obtained when one BALB/c foetal pancreas was transplanted under the kidney capsule of isogeneic diabetic recipient mice. Transplant recipients

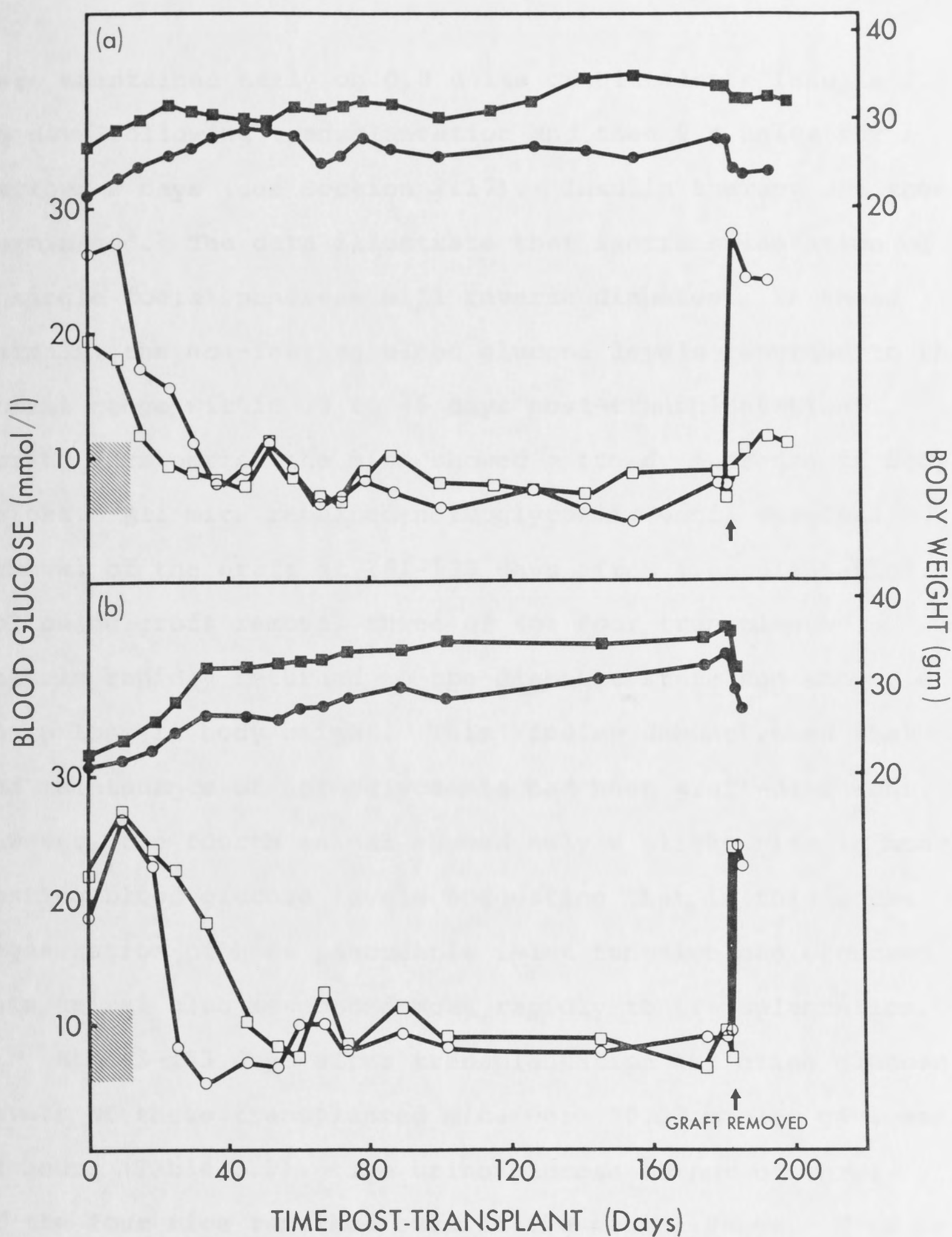


Figure 3.4 Non-fasting blood glucose levels (open symbols) and body weight (closed symbols) of diabetic BALB/c mice following isotransplantation of a single foetal pancreas under the kidney capsule. The shaded region defines the 95% probability interval for the non-fasting blood glucose levels of normal BALB/c mice. Grafts were removed by excision of the graft-bearing kidney. Each panel shows the data for two transplanted mice.

were maintained daily on 0.8 units of Ultralente insulin for 65 days following transplantation and then 0.4 units for a further 2 days (see Section 2.17). Insulin therapy was then terminated. The data illustrate that isotransplantation of a single foetal pancreas will reverse diabetes. In these animals, the non-fasting blood glucose levels returned to the normal range within 23 to 45 days post-transplantation. During this period the mice showed a steady increase in body weight. All mice remained normoglycaemic until surgical removal of the graft at 181-182 days after transplantation. Following graft removal three of the four transplanted animals rapidly returned to the diabetic state and showed a sharp loss in body weight. This finding demonstrated that the maintenance of normoglycaemia had been graft-dependent. However, the fourth animal showed only a slight rise in non-fasting blood glucose levels suggesting that, in this mouse, regeneration of host pancreatic islet function had occurred. This animal also responded most rapidly to transplantation.

At 143-175 days after transplantation the urine glucose levels of these transplanted mice were ≤ 0.07 mmoles glucose/24 hours (Table 3.2). The urine glucose output of three of the four mice remained outside the normal range. Even so, these levels were at least two orders of magnitude lower than for diabetic control mice.

Figure 3.5 shows the results obtained following isotransplantation of four foetal pancreases under the kidney capsule. These transplant recipients were maintained daily on 0.8 units of Ultralente insulin for 14 days after

TABLE 3.2

Urine glucose output following isotransplantation of one and four uncultured foetal pancreases

Isograft	Mouse Code	Time post-transplant (days)	Urine glucose ^a (mmoles/24hr)
1 foetal pancreas	(1)	143	0.03
		175	0.07
	(2)	143	0.02
		175	0.01
	(3)	154	0.07
		174	0.01
	(4) ^b	157	0.01
		174	0.00
4 foetal pancreases	(1)	141	0.26 ^c
		173	0.01
	(2)	142	0.01
		174	0.01
	(3)	142	0.01
		174	0.00
	(4)	142	0.00
		174	0.01
	(5)	142	0.09
		174	0.01
	(6)	159	0.01
		176	0.01

a. Urine glucose output for normal BALB/c mice was 0.00 mmoles glucose/24 hours; the levels for diabetic BALB/c mice were ≥ 9.38 mmoles glucose/24 hours (Section 2.18).

b. This mouse showed evidence of spontaneous recovery from diabetes following graft removal (Section 3.2.3).

c. Urine collection cylinder contained approximately 7 ml of loosely packed food grains (see Section 2.18).

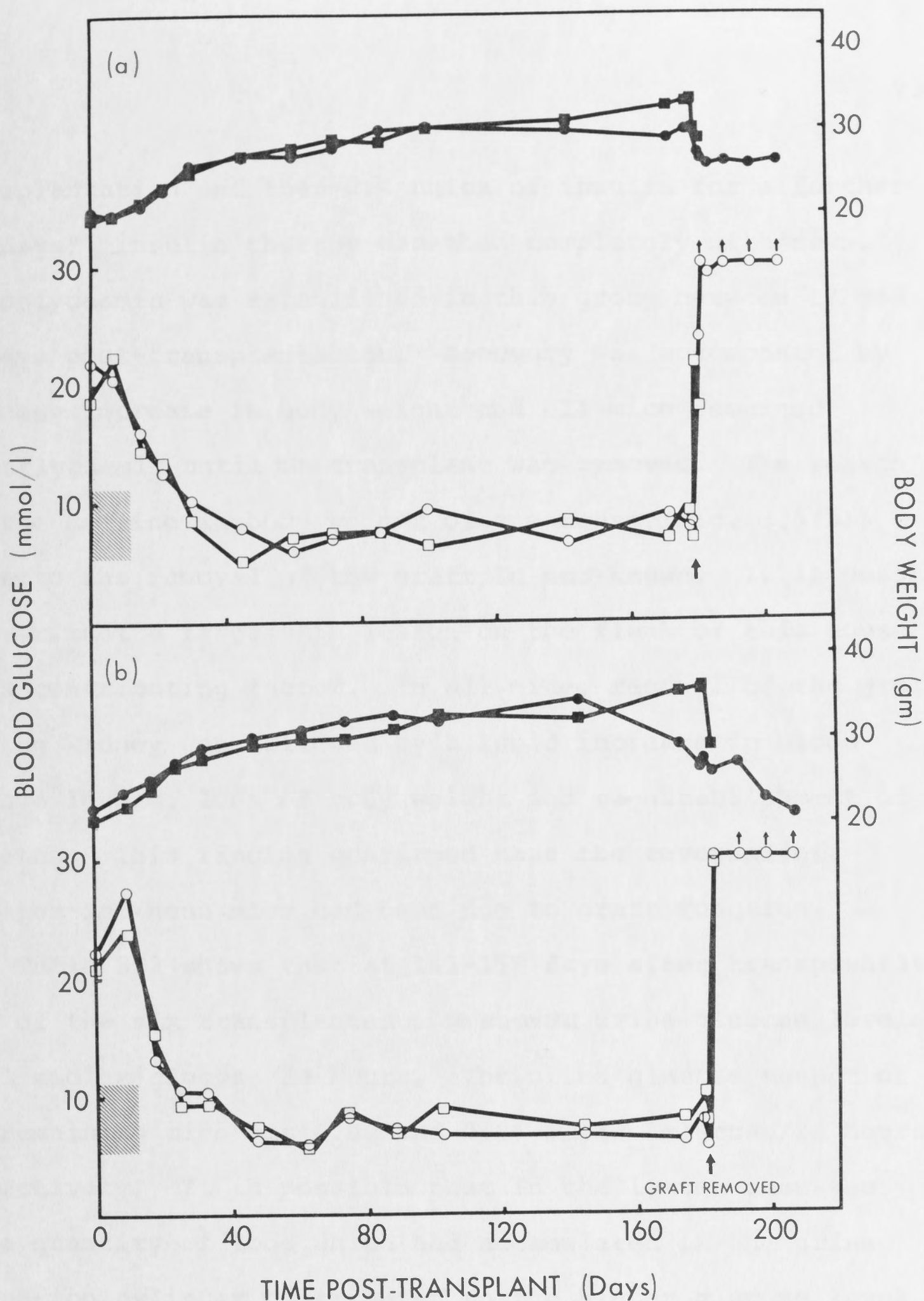


Figure 3.5 Non-fasting blood glucose levels (open symbols) and body weight (closed symbols) of Streptozotocin-treated diabetic BALB/c mice after transplantation of four isogeneic foetal pancreases beneath the kidney capsule. The shaded region represents the 95% probability interval for the non-fasting blood glucose levels of normal BALB/c mice. Grafts were removed by excision of the graft-bearing kidney. Each panel shows the data for 2 transplant recipients. The data are representative of a total group of six transplanted mice.

transplantation and then 0.4 units of insulin for a further two days; insulin therapy was then completely withdrawn. Normoglycaemia was established in this group between 17 and 30 days post-transplantation. Recovery was accompanied by a steady increase in body weight and all mice remained normoglycaemic until the transplant was removed. The reason for the decline in body weight of one mouse (Fig. 3.5(b)), prior to the removal of the graft, is not known. It is possible, however, that a large skin lesion on the flank of this mouse was a contributing factor. In all mice, removal of the graft-bearing kidney was followed by a rapid increase in blood glucose levels, loss of body weight and re-establishment of diabetes. This finding confirmed that the reversal of diabetes in these mice had been due to graft function.

Table 3.2 shows that at 141-159 days after transplantation four of the six transplanted mice showed urine glucose levels ≤ 0.01 mmoles glucose/24 hours. The urine glucose output of the remaining mice was 0.09 and 0.26 mmoles glucose/24 hours respectively. It is possible that in the latter case the large quantity of food which had accumulated in the urine collection cylinder contributed to the higher glucose level. By 173-176 days after transplantation, all transplant recipients showed urine glucose levels ≤ 0.01 mmoles glucose/24 hours. However, five of the six measurements remained outside the normal range. Although these transplanted mice were not completely aglycosuric, the urine glucose levels were approximately 3 orders of magnitude lower than for diabetic control mice.

This study demonstrated that a single isogeneic foetal pancreas transplanted under the kidney capsule will reverse diabetes in the mouse. Increasing the amount of donor tissue four-fold reduced the time required for re-establishment of normoglycaemia but this effect was only marginal.

3.2.4 Survival of foetal pancreas in organ culture

To establish the optimal procedure for the organ culture of foetal mouse pancreas in a high oxygen gas phase, two organ culture methods were compared: the submerged organ culture method and the grid organ culture technique. In general, submerged cultures (Section 2.3.1) contained sufficient medium for the meniscus to just cover the tissue surface. In grid cultures (Section 2.3.2) the foetal pancreas tissue was directly exposed to the high oxygen atmosphere.

3.2.4.1 Organ culture of whole foetal pancreas

After organ culture of whole foetal pancreas (Section 2.3.1; Section 2.3.2) for 10 days, most of the acinar tissue had disappeared and only remnants of acini were observed. The major component of foetal pancreas tissue present was undifferentiated tissue. In addition, some duct epithelium and a small number of aldehyde fuchsin-positive islets were identified. Some groups of aldehyde fuchsin-staining cells were also found scattered throughout the undifferentiated tissue compartment. Table 3.3 shows that two of two grid-cultured foetal pancreases and four of five submerged-cultured foetal pancreases contained some viable pancreatic tissue and necrotic tissue (Fig. 3.6). The

TABLE 3.3

Survival of 10 day-cultured whole foetal mouse pancreas

Culture method	Total No. pancreases	Histological score ^a /pancreas
Grid	2	+
		++
Submerged	5	+
		+
		+
		++
		+++

a. Histological score key:

- +++ Viable pancreatic tissue and no necrosis.
- ++ Viable pancreatic tissue and some necrotic tissue.
- + Extensive necrosis and some viable pancreatic tissue.

Figure 3.6. Histological sections of whole foetal mouse pancreas following organ culture for 10 days in 95% O₂, 5% CO₂. (a) Submerged culture. Haematoxylin and eosin, x 183. (b) Grid culture. Haematoxylin and eosin, x 179. Note tissue necrosis shown by pyknotic nuclei (arrow).

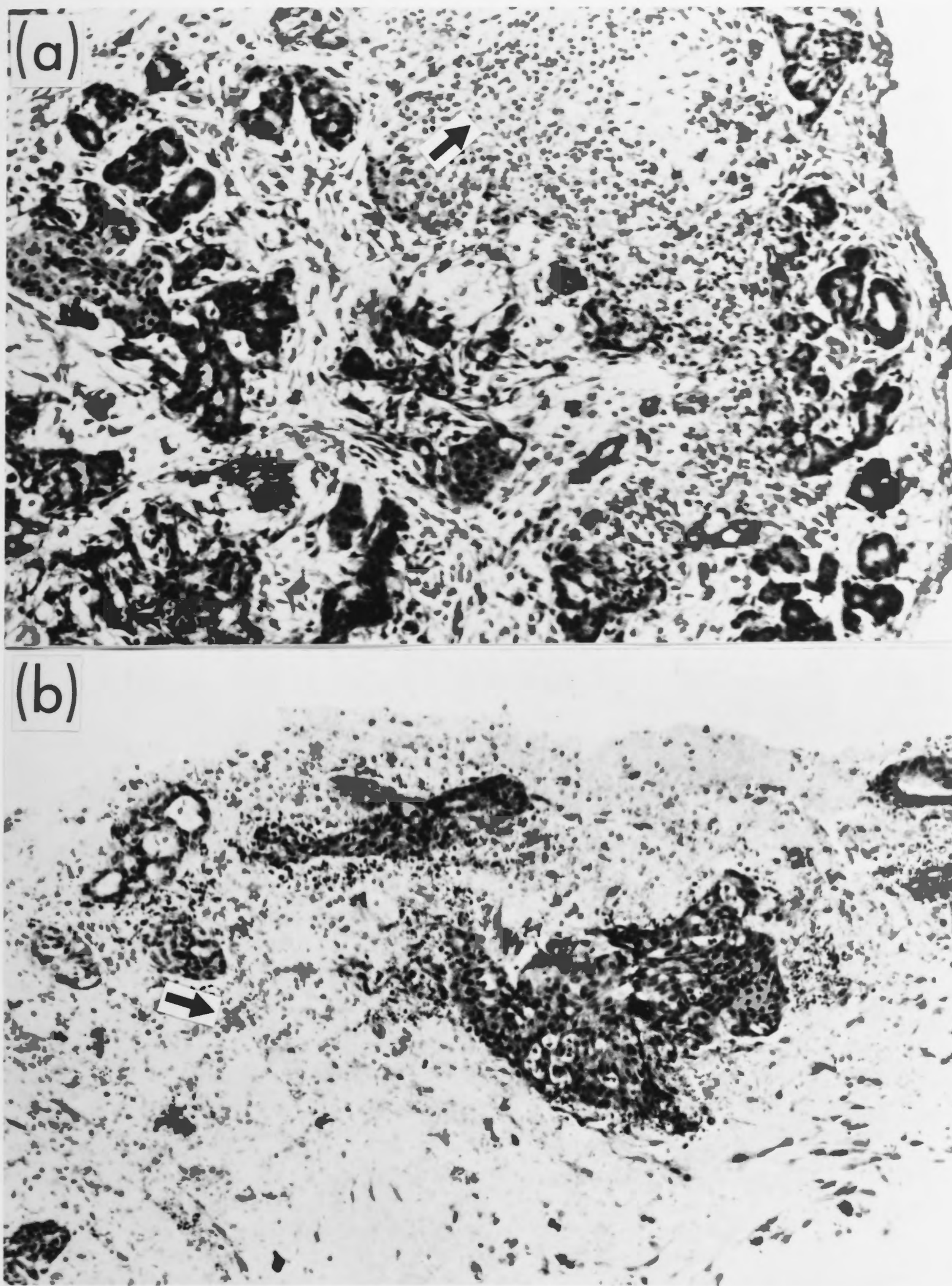


Figure 3.6 Histological sections of whole foetal mouse pancreas following organ culture for 10 days in 95% O₂, 5% CO₂ (a) Submerged culture. Haematoxylin and eosin, x 183. (b) Grid culture. Haematoxylin and eosin, x 179. Note tissue necrosis shown by pycnotic nuclei (arrow).

remaining cultured pancreas contained well-preserved pancreatic tissue and some fibrotic tissue. Tissue necrosis was commonly found in the central region of submerged-cultured foetal pancreases and was characterized by pycnotic cells. Both peripheral and central necrosis were identified in grid cultures. Macroscopically, the submerged cultures had condensed into a compact mass by 10 days. It is likely, therefore, that the central necrosis identified by histological examination resulted from inadequate diffusion of oxygen and possibly other nutrients. In contrast, the peripheral necrosis of grid cultures suggested that direct exposure to a high oxygen gas phase was toxic for foetal pancreas tissue.

In an attempt to improve the recovery of viable pancreatic tissue, in subsequent experiments the foetal pancreas was cut into three segments prior to organ culture.

3.2.4.2 Organ culture of foetal pancreas segments

Table 3.4 shows the survival of foetal pancreas segments following organ culture in 95% O₂, 5% CO₂ for 10 and 17 days. At 10 days, four of ten grid-cultured segments and sixteen of forty-two submerged-cultured segments showed good survival of foetal pancreas tissue with either little or no necrosis or fibrosis. This finding suggested that, compared to whole foetal pancreas cultures (Table 3.3), the division of foetal pancreases into segments marginally improved the survival of pancreatic tissue. However, statistical analyses showed that by 17 days there was a significant decline in the survival of foetal pancreas tissue cultured on grids

TABLE 3.4

Survival of foetal mouse pancreas segments in organ culture

Culture method	Duration of culture (days)	Total No. segments	No. segments with histological score ^a				
			++++	+++	++	+	0
Grid	10	10	2	2	2	2	2 ^b
	17	7	-	-	-	-	7 ^{b,c}
Submerged	10	42	4	12	12	10 ^d	4
	17	38	-	8	6	21 ^d	3 ^c

a. Histological score key:

++++ Viable pancreatic tissue only.

+++ Extensive proportion of viable pancreatic tissue but some necrosis or fibrotic connective tissue.

++ Viable pancreatic tissue and an approximately equal proportion of necrotic or fibrotic tissue.

+ Extensive necrosis or fibrosis and small quantity of viable pancreatic tissue.

0 Necrotic or fibrotic connective tissue with only remnants of pancreatic tissue.

b. $P = 0.0037$ (Exact probability test; Swinscow, 1980b).

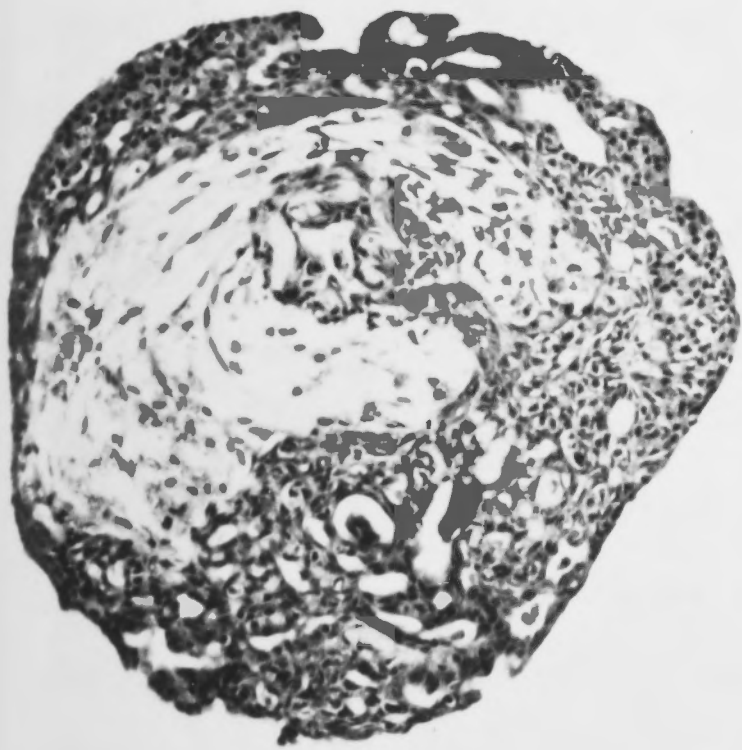
c. $P = 0.0001$ (Exact probability test: Swinscow, 1980b).

d. $P < 0.01$ (Approximation to exact probability test; Kendall and Stuart, 1973).

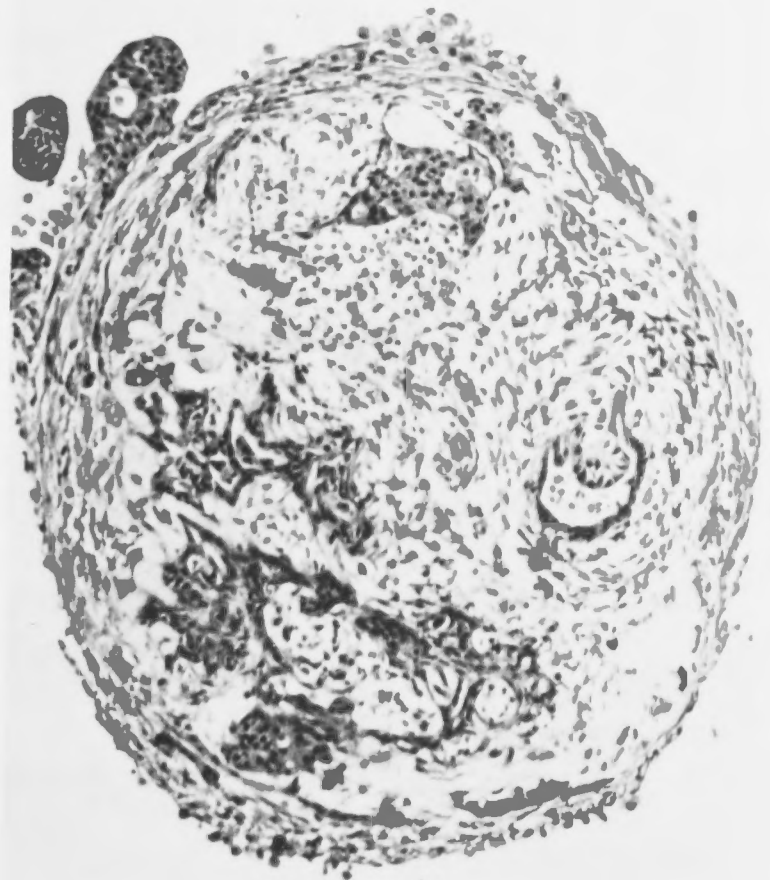
($P = 0.0037$). Whereas only two of ten segments showed no evidence of viable pancreatic tissue after 10 days of grid culture, seven of seven segments were necrotic by 17 days (Fig. 3.7(c)). Submerged cultures showed significantly better survival than grid cultures at 17 days ($P = 0.0001$); only three of thirty-eight submerged-cultured segments failed to show signs of viable pancreatic tissue. The remaining 17 day-cultured segments contained undifferentiated tissue, a small amount of differentiated tissue, some duct epithelium and fibrotic connective tissue or some necrotic tissue (Fig. 3.7(a)). No acinar tissue was identified and it is likely that the degenerated exocrine compartment was replaced by fibrotic connective tissue. Generally, only cells located on the periphery of differentiated islet-like tissue were stained with aldehyde fuchsin. Whether the scant aldehyde fuchsin-positive staining was due to incomplete differentiation of the islet tissue or to beta cell degranulation in response to glucose (2 gm/litre) and/or nicotinamide (1 mg/litre) present in RPMI 1640 culture medium (Moore *et al.*, 1967; Andersson and Hellerström, 1980) cannot be distinguished. Table 3.4 also shows that, compared to 10 day-submerged cultures, a significantly larger proportion of 17 day-submerged cultures showed extensive necrosis or fibrosis with little viable pancreas tissue ($P < 0.01$; Fig. 3.7(b)).

These findings demonstrate that the submerged organ culture procedure permits long-term culture of foetal mouse pancreas in a 95% O_2 , 5% CO_2 gas phase. However, the decline in the recovery of viable tissue by 17 days indicates that prolonged organ culture damages foetal islet tissue. In

(a)



(b)



(c)



Figure 3.7 Histological appearance of foetal mouse pancreas segments following organ culture for 17 days in 95% O₂, 5% CO₂. (a) Submerged culture shows viable pancreatic tissue and some necrosis. Haematoxylin and eosin, x 187. (b) Submerged-cultured segment showing widespread fibrosis, some necrosis and small amount of viable pancreatic tissue. Note islet-like tissue on periphery of segment. Haematoxylin and eosin, x 129. (c) Grid-cultured segment composed entirely of necrotic tissue. Haematoxylin and eosin, x 140.

contrast, the grid culture technique failed to maintain foetal pancreas *in vitro* for 17 days. It is likely that this failure was attributable to direct oxygen toxicity.

3.2.5 Islet survival after organ culture and isotransplantation

The effect of prolonged organ culture on islet differentiation and survival was examined by determining the capacity of cultured foetal pancreas to develop histologically recognizable islets following isotransplantation. Table 3.5 shows that following culture for 10 days islet survival was observed in all transplants. After 17 days of organ culture, two of twenty-one isografts contained remnants of islets which stained with aldehyde fuchsin; these grafts predominantly consisted of fibrotic connective tissue. The remaining isografts were mainly composed of well-differentiated islets and connective tissue. In addition, duct epithelium, a small quantity of adipose tissue and some necrotic tissue were observed. In one isograft some residual acinar tissue was also identified.

After a 20 day culture period, three of nineteen isografts showed no islet survival; these transplants were composed entirely of connective tissue elements and some necrotic tissue. The remaining isografts contained well vascularized, fully differentiated islets which stained with aldehyde fuchsin (Fig. 3.8). There was also evidence of fibrotic tissue and occasionally small amounts of adipose tissue and necrotic tissue. The quantity of adipose tissue present in cultured isografts was substantially less than that

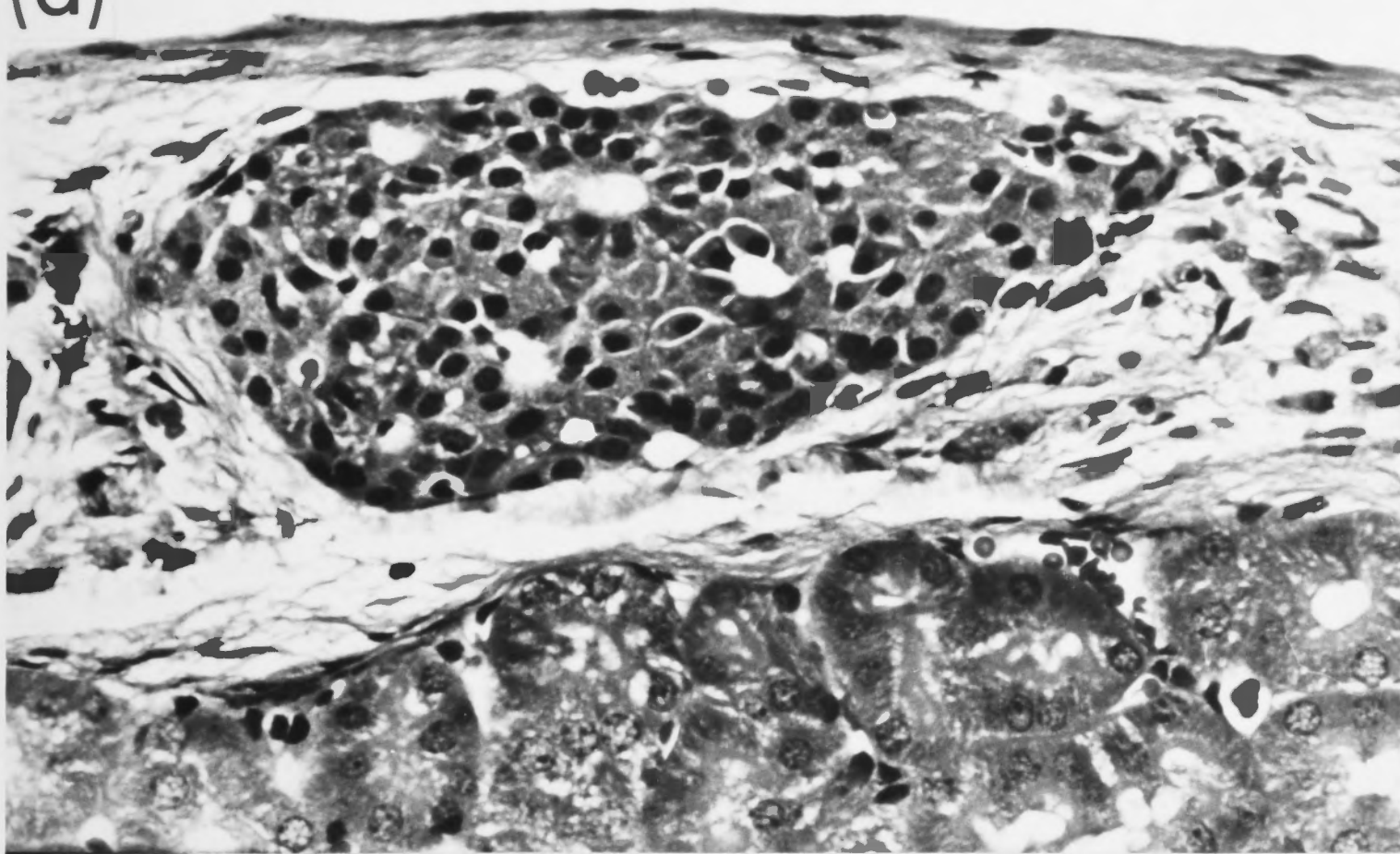
TABLE 3.5

Survival of islet tissue after submerged organ culture and isotransplantation under the kidney capsule^a.

Time in culture (days)	No. transplants examined ^b	No. transplants showing different- iated islets	No. transplants without islet development	% transplants showing islet survival
0	12	12	-	100
10	12	12	-	100
17	21	19	2 ^c	90
20	19	16	3 ^d	84

- a.* Cultured isografts consisted of a single foetal pancreas segment; uncultured isografts comprised one whole foetal pancreas.
- b.* Tissue was examined 2 - 4 weeks after transplantation.
- c.* Two of 21 isografts contained islet remnants and consisted mainly of fibrotic tissue.
- d.* Three of 19 isografts were composed entirely of fibrotic and necrotic tissue.

(a)



(b)

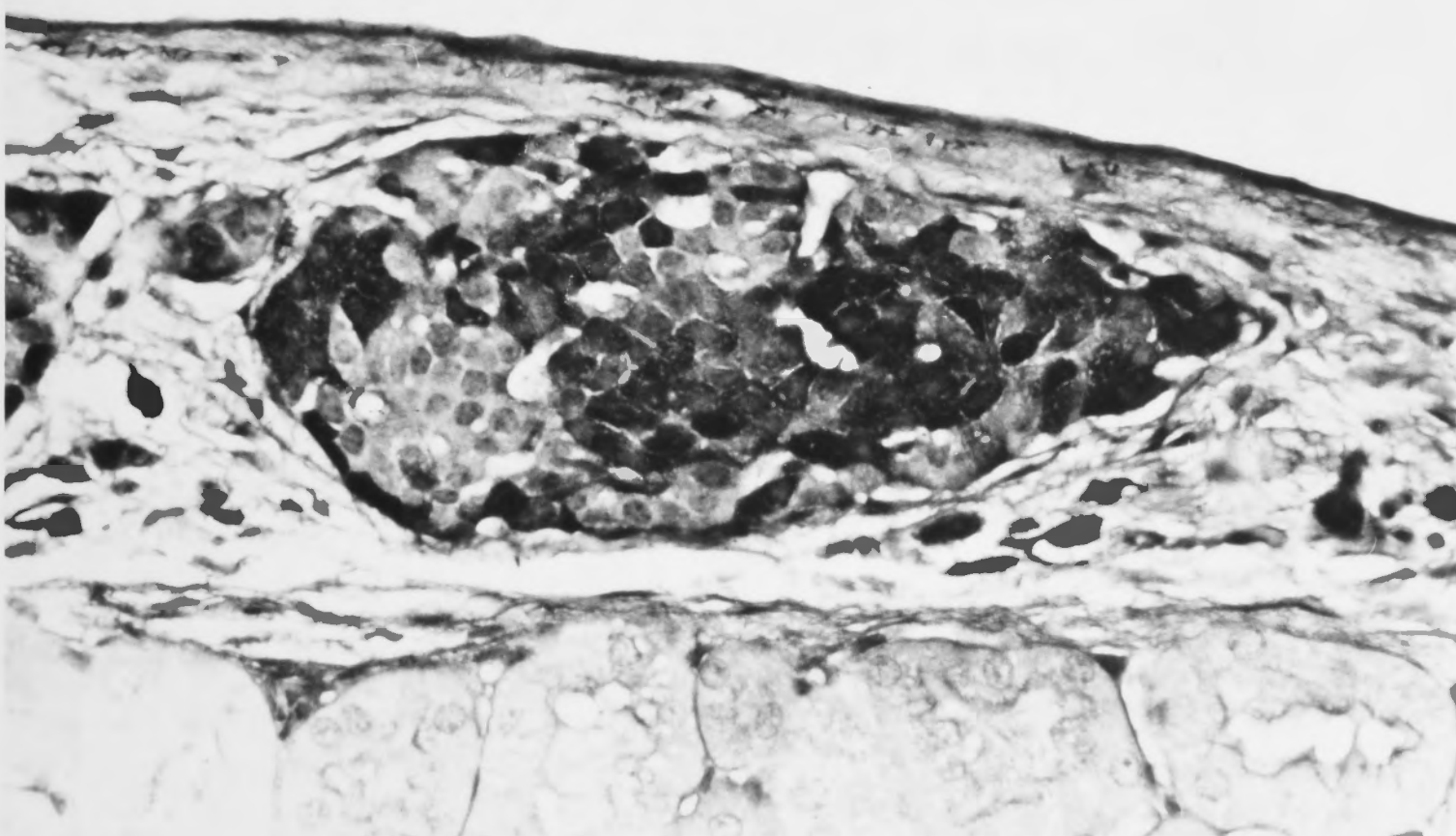


Figure 3.8 Isograft of a single 20 day-cultured foetal pancreas segment 4 weeks after transplantation under the kidney capsule of a BALB/c recipient mouse (a) Note well developed islet tissue. Haematoxylin and eosin, x 426. (b) Note presence of darkly stained beta cells. Aldehyde fuchsin, x 400.

observed in uncultured isografts. This finding may be attributable to the loss of adipocytes during organ culture. No acinar tissue was observed in 20 day-cultured isografts.

In contrast to uncultured isografts (Section 3.2.2), no lymph-node-like structures were observed in cultured isografts. This finding suggests that organ culture in a high oxygen atmosphere substantially damages the primitive lymphoid component of mouse foetal pancreas. There was no qualitative difference in islet development between uncultured and 10 day-cultured isografts. However, the data indicate that islet survival was impaired following 17 - 20 days of organ culture. These findings support the proposal that prolonged culture in an oxygen rich atmosphere can damage primitive foetal islet tissue (Section 3.2.4.2).

3.2.6 Function of 20 day-cultured foetal pancreas tissue

It is apparent from the above data (Section 3.2.5) that the limit of foetal pancreas survival in a 95% O₂, 5% CO₂ gas phase is approximately 17 to 20 days. Furthermore, studies on the function of uncultured foetal pancreas isografts indicated that 1 to 4 foetal pancreases will reverse diabetes (Section 3.2.3). To examine the functional capacity of transplanted 20 day-cultured tissue, tissue from two foetal pancreases (six segments), cultured for 20 days, was transplanted under the kidney capsule of isogeneic diabetic recipients.

Figure 3.9 shows that 20 day-cultured isografts can reverse diabetes. The data are representative of a total group of six transplanted mice. Transplant recipients were treated

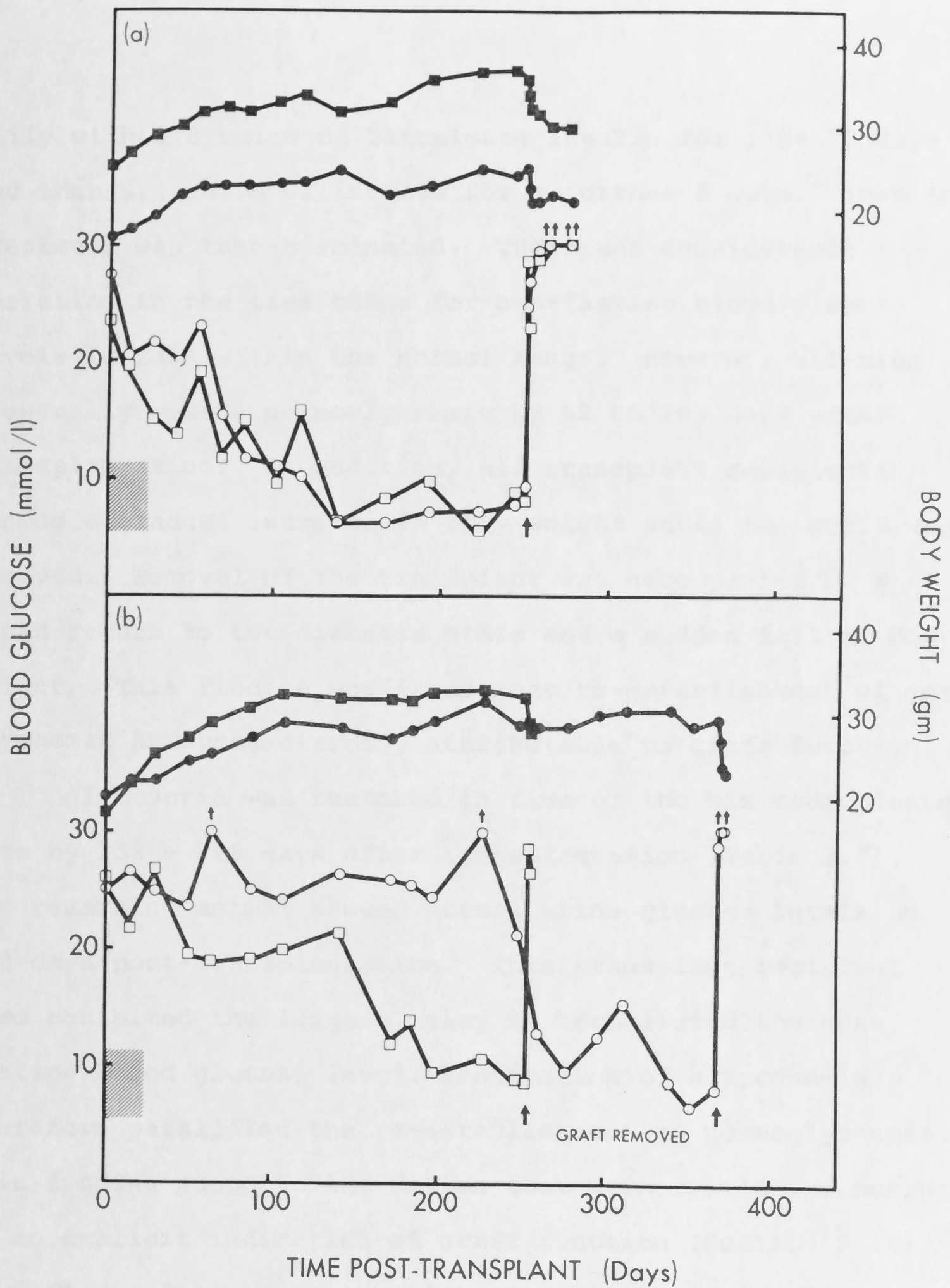


Figure 3.9 Non-fasting blood glucose levels (open symbols) and body weight (closed symbols) of diabetic BALB/c mice carrying isografts of six 20 day-cultured foetal pancreas segments (two 20 day-cultured foetal pancreases) under the kidney capsule. Each panel shows the data for two transplanted mice. The shaded region defines the 95% probability interval for the non-fasting blood glucose levels of normal BALB/c mice. Grafts were removed by excision of the graft-bearing kidney.

daily with 0.8 units of Ultralente insulin for 118-127 days and then 0.4 units of insulin for a further 2 days. Insulin treatment was then terminated. There was considerable variation in the time taken for non-fasting blood glucose levels to fall within the normal range. However, all mice eventually became normoglycaemic by 62 to 269 days after transplantation. In addition, all transplant recipients showed a gradual increase in body weight until the graft was removed. Removal of the transplant was accompanied by a rapid return to the diabetic state and a sudden fall in body weight. This finding confirmed that re-establishment of normoglycaemia had been directly attributable to graft function.

Aglycosuria was restored in five of the six transplanted mice by 238 - 248 days after transplantation (Table 3.6). The remaining animal showed normal urine glucose levels by 338 days post-transplantation. This transplant recipient also exhibited the longest delay in normalizing the non-fasting blood glucose level. Restoration of aglycosuria therefore paralleled the re-establishment of normoglycaemia. This finding supports the notion that urinary glucose output is an explicit indication of graft function (Section 2.18).

Figure 3.10 shows the histological appearance of one of the six 20 day-cultured isografts which successfully reversed Streptozotocin-induced diabetes. At 265 days after transplantation, the graft consisted of massive, well-differentiated islets which stained intensely with aldehyde fuchsin.

This study demonstrated that two 20 day-cultured foetal pancreases can reverse diabetes. However it is apparent that organ culture damages the function of foetal pancreas

TABLE 3.6

Urine glucose output following isotransplantation of 20 day-
cultured foetal pancreas tissue

Mouse Code	Time post-transplant (days)	Urine glucose ^a (mmoles/24 hr)
(1)	193	0.02
	238	0.00
(2)	193	0.02
	238	0.00
(3)	194	0.01
	239	0.00
(4)	172	0.01
	186	0.03
	248	0.00
(5)	172	1.25
	186	0.01
	248	0.00
(6)	172	9.68
	186	15.30
	248	0.95
	338	0.00
	343	0.00

^a. Urine glucose output for normal BALB/c mice was 0.00 mmoles glucose/24 hours; urine glucose levels for control diabetic BALB/c mice were ≥ 9.38 mmoles glucose/24 hours (Section 2.18).

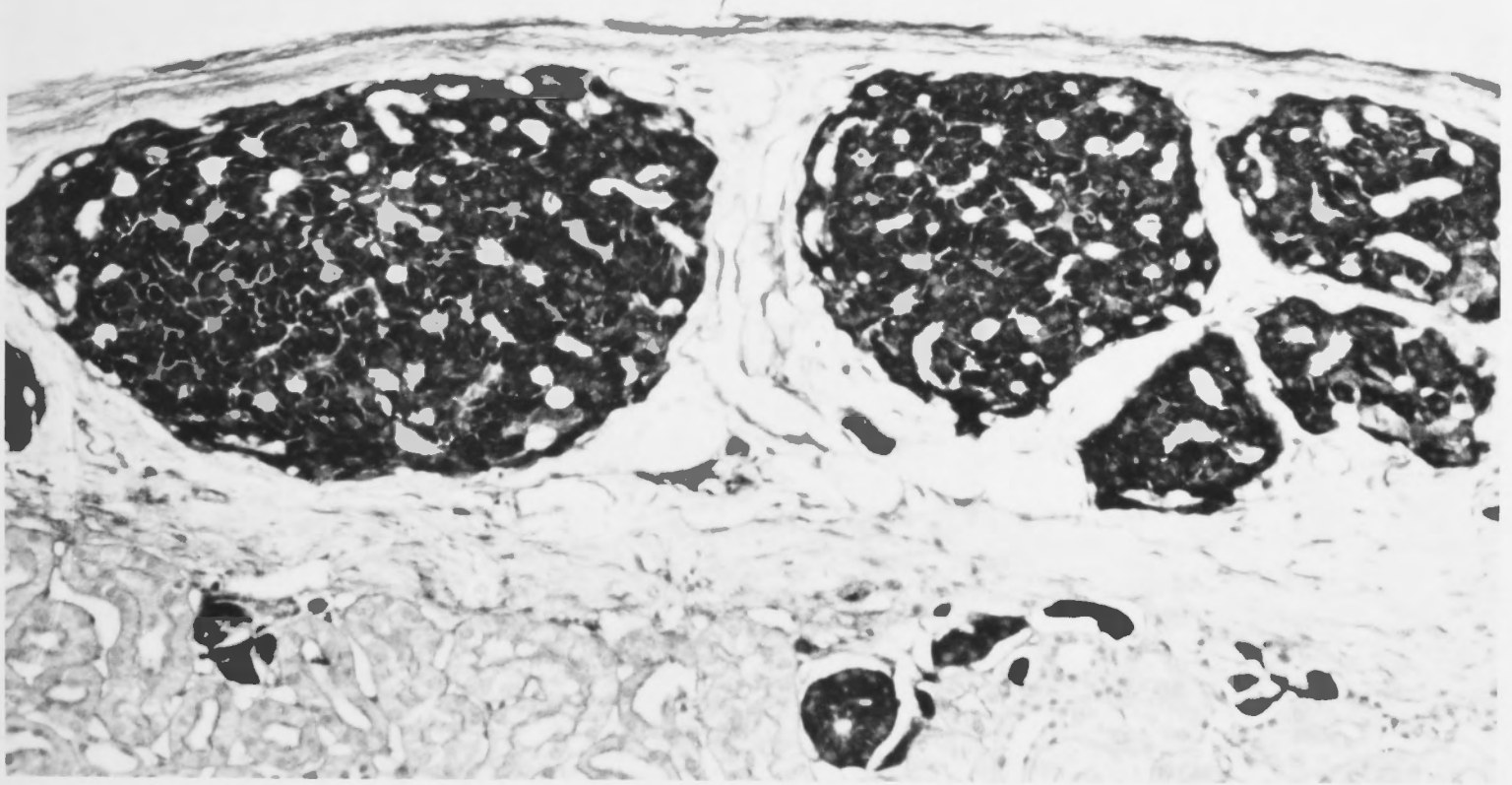


Figure 3.10 Isograft of six 20 day-cultured foetal BALB/c pancreas segments 265 days after transplantation under the kidney capsule of a diabetic BALB/c recipient mouse. Note large differentiated islets with dense beta cell granulation. Aldehyde fuchsin, x 167.

tissue; isografts of two 20 day-cultured foetal pancreases were less efficient than isografts of one uncultured foetal pancreas (Section 3.2.3) in reversing diabetes. Other data presented in this chapter support the notion that the impaired functional capacity of 20 day-cultured foetal pancreas tissue is due to the decline in survival of primitive islet tissue following long-term organ culture (Section 3.2.4.2, Section 3.2.5).

3.3 DISCUSSION

The selective growth and development of endocrine tissue and the atrophy of exocrine tissue are characteristic properties of foetal pancreas isografts which have been well documented (Browning and Resnik, 1951-1952; Coupland, 1960; Hegre *et al.*, 1976a; Brown *et al.*, 1976; McEvoy and Hegre, 1978). In this chapter we confirmed these properties in isografts of mouse foetal pancreas transplanted beneath the kidney capsule.

In addition, we presented histological evidence for the contamination of foetal mouse pancreas explants with primitive lymphoid tissue. The associated lymphoid component was not found within the pancreas itself but around the diffuse periphery of the organ. Further support for the existence of lymphoid contamination was provided by the presence of lymph-node-like structures within foetal pancreas isografts. It should be noted that the primitive lymphoid tissue was not distinguishable at the stereomicroscopic level and therefore could not be selectively removed from explants prior to transplantation. Thus, the lymphoid structures which developed in isografts probably resulted from the co-transplantation of

associated lymphoid elements. In view of the resemblance of these structures to organized lymph nodes, the primitive lymphoid component of foetal mouse pancreas may represent developing lymph nodes derived from the mesentery which surrounds the organ *in situ*. The significance of these findings relates to the possible contribution of such an appendage of lymphoid cells to the immunogenicity of foetal pancreas tissue (see Chapter 4).

Subsequent studies in our laboratory have demonstrated that the associated lymphoid component of foetal pancreas tissue is not unique to the murine organ but instead represents a general phenomenon. Recently, primitive lymphoid tissue has been identified also in histological sections of foetal pig and foetal human pancreas (K.J. Lafferty, personal communication). The lymphoid component of human foetal pancreas has been analysed using a range of mouse anti-human monoclonal antibodies and the immunoperoxidase staining technique. Thomson and colleagues (1982) found that 60-70% of the cells comprising the associated lymphoid component were leukocytes; of these cells, approximately 60% stained with anti-*Ia*-like monoclonal antibody and 10-30% were identified as T lymphocytes. The cell populations expressing class II antigens (Section 1.3) included B cells, macrophages and dendritic cells. Similarly, Danilovs *et al.* (1982) have identified class II antigen-bearing cells and T cells in the peripheral lymphoid tissue of human foetal pancreas explants. No such analysis has yet been made of the lymphoid tissue associated with mouse foetal pancreas. However, it is not unlikely that similar cell populations would be represented.

We have demonstrated that isografts of a single foetal mouse pancreas transplanted under the kidney capsule can reverse Streptozotocin-induced diabetes in recipient mice. Other groups have also shown that isotransplantation of a single foetal pancreas is sufficient to reverse experimentally-induced diabetes (Brown *et al.*, 1981; Hoffman *et al.*, 1981). These findings are important because they illustrate that a particular advantage of the use of foetal pancreas transplants for the reversal of diabetes is the potential for a single foetal donor to provide sufficient islet tissue to normalize the blood glucose levels of a diabetic host. In contrast, multiple donors are required to isolate sufficient numbers of adult islets for the reversal of diabetes following transplantation to individual recipients (Gray and Watkins, 1976a; Feldman *et al.*, 1977; Bowen and Lafferty, 1980). It is likely that the difference in the number of foetal and adult donors required for transplantation can be attributed to the loss of adult islet tissue during the islet isolation procedure and to the growth potential of primitive foetal islet tissue.

The administration of insulin to recipients carrying isografts consisting of eight foetal rat pancreases has been shown to promote the growth and differentiation of the beta cell component of the foetal endocrine tissue (McEvoy and Hegre, 1978, 1979) and to enhance graft function (McEvoy *et al.*, 1978; McEvoy and Hegre, 1979) in a proportion of transplanted rats. Support for the former observation has been provided by *in vitro* studies; the addition of exogenous insulin to cultures of foetal rat pancreas has been shown to increase

markedly both the volume of the beta cell component and the insulin content of the explants (McEvoy, 1981). The mechanism of insulin action on beta cell growth, however, is not understood. Hoffman *et al.* (1981) reported that the function of isografts of a single foetal mouse pancreas transplanted to the spleen was improved in the long-term by treatment of transplant recipients with parenteral insulin. In our studies, recipient mice were also maintained daily on insulin. Whether this treatment enhanced the growth and differentiation of the foetal islet tissue is not known.

Chase *et al.* (1979) reported that foetal mouse pancreas was particularly sensitive to oxygen toxicity and did not survive organ culture in 95% O₂, 5% CO₂ beyond 4 days. The results presented in this chapter show that the foetal mouse pancreas can be cultured in an oxygen rich atmosphere for up to 20 days and will function when subsequently transplanted to diabetic recipient mice. These conflicting results can be related to differences in culture technique. Chase and co-workers (1979) cultured tissue on stainless steel grids at the medium/gas interface. In preliminary experiments we used a similar culture procedure and also found a substantial deterioration in tissue viability. However, in our hands, this effect was seen after 10 days. The failure of foetal pancreas to survive prolonged culture was probably attributable to acute oxygen toxicity resulting from direct exposure of grid cultures to the high oxygen atmosphere. We obtained much better survival of foetal pancreas in culture when the tissue was submerged in a thin layer of medium. Histological examination of foetal pancreas tissue transplanted after 17

to 20 days of culture showed good islet development in 84-90% of the isografts; the remaining isografts showed evidence of a decline in islet survival. The submerged culture technique can therefore maintain foetal mouse pancreas in a high oxygen gas phase for up to 20 days.

In addition, we have demonstrated that foetal pancreas tissue retains the capacity to function after prolonged organ culture. However, isografts of two 20 day-cultured foetal pancreases did not function as efficiently as isografts of a single uncultured foetal pancreas in reversing Streptozotocin-induced diabetes. The histological studies detailed in this chapter indicate that this deterioration in graft function was probably related to the loss of endocrine tissue during prolonged culture. Other investigators have also demonstrated that the ability of cultured foetal pancreas isografts to reverse Streptozotocin-induced diabetes can be influenced by the culture conditions. Using a 5% CO₂, 95% air gas phase, Mandel and colleagues (1980) reported some reduction in the functional capacity of foetal mouse pancreas isografts following organ culture in the presence of a high glucose concentration (4gm/litre) in the culture medium.

In our study of the function of cultured isografts, the eventual recovery from diabetes served to illustrate the growth potential and regenerative capacity of the endocrine component of cultured foetal pancreas tissue. Indeed, the apparent capacity of ductal elements to differentiate into endocrine tissue (Coupland, 1960; Bunnag, 1966; Hegre *et al.*, 1976b) suggests that the preservation of duct epithelium following long-term organ culture (Section 3.2.4.2)

may contribute to the growth of foetal islet tissue following transplantation (Hegre *et al.*, 1976b; Jonasson and Hoversten, 1978). Furthermore, transplant recipients were maintained on insulin for 120-129 days after transplantation. It is possible, therefore, that the insulin treatment enhanced islet growth and differentiation (McEvoy *et al.*, 1978; McEvoy and Hegre, 1978, 1979). In any case, insulin therapy permitted these mice to be maintained for the considerable length of time required for graft function to develop and return the animals to normoglycaemia.

The absence of lymph-node-like structures in cultured isografts suggests that the organ culture procedure damaged the primitive lymphoid tissue associated with mouse foetal pancreas. This finding is significant because it supports the proposal that organ culture in a 95% O₂, 5% CO₂ gas phase removes passenger blood cells from donor tissue prior to transplantation (Lafferty *et al.*, 1976b; Section 1.6.3). The effectiveness of organ culture in modulating foetal pancreas immunogenicity will be examined in the following chapter.

3.4 SUMMARY

In this chapter we presented evidence indicating that foetal mouse pancreas is contaminated with primitive lymphoid tissue. In addition, isografts of a single foetal mouse pancreas transplanted beneath the kidney capsule of diabetic recipients were shown to reverse Streptozotocin-induced diabetes.

An organ culture procedure was described for the maintenance of foetal pancreas tissue in a high oxygen atmosphere for prolonged periods. Following organ culture for 20 days, foetal pancreas tissue retained the functional capacity to reverse diabetes in transplanted mice.

In the following chapter we will assess the effectiveness of this organ culture procedure in reducing the immunogenicity of foetal pancreas allografts. The investigation includes a comparative study of the effect of the same organ culture system on the allotransplantation of isolated adult mouse islets.

4.1 INTRODUCTION

Reports in the 1950-1970s demonstrated that culture of donor tissue prior to transplantation could prolong survival of allogeneic mouse isografts (Lafferty, 1974; 1975a; Section 1.6.7) and rat islets (Jacob, 1974; Langer and Chappman, 1974; Section 1.6.3). Following this demonstration, several groups adopted the organ culture technique for the allotransplantation of fetal and adult pancreatic islet tissue.

CHAPTER 4

In the case of fetal pancreas tissue, little or no prolongation of allograft survival was observed (Lafferty, 1975a; 1975b).

THE EFFECT OF ORGAN CULTURE ON THE IMMUNOGENICITY

OF FOETAL MOUSE PANCREAS

AND ISOLATED ADULT MOUSE ISLETS

It is well known that the immunogenicity of an allograft is related to the degree of processing of the antigenic material. In the case of pancreatic islets, it has been shown (Lafferty, 1975a; 1975b) that islets cultured for 21 days in 5% CO₂ air failed to prolong the survival of islet allografts in the rat.

In contrast, several groups working with isolated adult islets reported either partial or complete success. Langer (1977) obtained partial reversal of diabetes with allografts of rat islets cultured for 4 days in 5% CO₂ air. Lafferty and colleagues (1975a) were only able to demonstrate prolonged survival of allogeneic rat islets cultured for 7 days in a gas phase of 5% CO₂, 95% air when the transplant recipients were given a single dose of rabbit anti-rat lymphocyte serum (ALS) at the time of transplantation. Lafferty (1979) reported some evidence suggesting that organ culture of

4.1 INTRODUCTION

Reports in the mid-1970s demonstrated that organ culture of donor tissue prior to transplantation prolonged the survival of allogeneic mouse thyroid (Lafferty *et al.*, 1975, 1976a; Section 1.6.3) and ovary (Jacobs, 1974; Lueker and Sharpton, 1974; Section 1.6.3). Following this demonstration, several groups adopted the organ culture technique for the allotransplantation of both foetal and adult pancreatic islet tissue.

In the case of foetal pancreas tissue, little or no prolongation of allograft survival was observed. Hegre *et al.* (1976a) demonstrated that allografts of foetal rat pancreas cultured for 10 days in a gas phase of 95% O₂, 5% CO₂ were acutely rejected. Mandel and Higginbotham (1979) reported that long-term culture in a 5% CO₂, 95% air atmosphere only delayed the rejection of foetal mouse pancreas allografts. In addition, Garvey and co-workers (1980b) showed that organ culture for 21 days in the same gas phase failed to prolong the survival of foetal pancreas allografts in the rat.

In contrast, several groups working with isolated adult islets reported either partial or complete success. Kedingner *et al.* (1977) obtained partial reversal of diabetes with allografts of rat islets cultured for 4 days in 5% CO₂, 95% air. Lacy and colleagues (1979a) were only able to demonstrate prolonged survival of allogeneic rat islets cultured for 7 days in a gas phase of 5% CO₂, 95% air when the transplant recipients were given a single dose of rabbit anti-rat lymphocyte serum (ALS) at the time of transplantation. Andersson (1979) reported some evidence suggesting that organ culture of

adult mouse islets for 4 weeks in a 5% CO₂, 95% air atmosphere improved the function of islet allografts. In our laboratory, a procedure was developed for the organ culture of isolated adult mouse islets in a high oxygen gas phase (Bowen *et al.*, 1980). Organ culture for 7 to 12 days in this gas phase facilitated the long-term survival of islets prepared from cyclophosphamide-pretreated donors. However there was no significant difference in the survival of 7 day-cultured allogeneic islets isolated from cyclophosphamide-pretreated and untreated donors, at 2 weeks post-transplantation (Bowen *et al.*, 1980).

Thus, there is controversy in the literature concerning the capacity for organ culture to facilitate the acceptance of islet tissue allografts.

In this chapter, we utilize the submerged organ culture procedure presented in Chapter 3 to examine the effect of culture in a high oxygen atmosphere on the immunogenicity of foetal mouse pancreas allografts. In essence, this technique successfully prolonged the survival of allografts of isolated adult mouse islets (Bowen *et al.*, 1980). We extend this study of the immunogenicity of isolated adult islets and compare the effectiveness of the same organ culture system on the survival of allografts of foetal pancreas and isolated adult islets transplanted across the same major histocompatibility barrier. We examine the functional capacity of uncultured and cultured foetal pancreas allografts to reverse Streptozotocin-induced diabetes. Finally, we investigate the combined effect of organ culture and recipient immunosuppression on promoting foetal pancreas allograft acceptance.

4.2 RESULTS

4.2.1 Allotransplantation of uncultured foetal pancreas

Allografts of uncultured BALB/c foetal mouse pancreas transplanted across a major histocompatibility barrier to CBA recipients were acutely rejected (see Table 4.1). At 2 weeks after transplantation, the grafts consisted mainly of scar tissue with some mononuclear cell infiltration (Fig. 4.1). There was no evidence of pancreatic islet tissue remaining and haemorrhage was frequently observed at the graft site.

This finding is consistent with reports from other groups (Garvey *et al.*, 1979a, 1980b; Millard *et al.*, 1980; Mullen and Shintaku, 1980) that uncultured foetal pancreas is immunogenic and evokes a vigorous allograft reaction following transplantation under the kidney capsule of recipient animals.

4.2.2 Effect of organ culture on foetal pancreas allograft survival

We set out to determine whether organ culture in a high oxygen gas phase would facilitate foetal pancreas allograft survival. In Chapter 3 we demonstrated that foetal mouse pancreas can survive in a 95% O₂, 5% CO₂ gas phase for up to 20 days. On this basis we examined the integrity of allografts of a single BALB/c foetal pancreas segment cultured in 95% O₂, 5% CO₂ for 10, 17 and 20 days prior to transplantation across a major histocompatibility barrier to CBA recipient mice.

Table 4.1 shows that all of eleven 10 day-cultured foetal pancreas allografts examined at 2 weeks after

TABLE 4.1

Effect of organ culture on foetal pancreas allograft survival

Duration of culture (days)	Time post- transplant (weeks)	Total No. grafts ^a examined	No. grafts with histological score ^b					% grafts surviving ^c
			++++	+++	++	+	0	
0	2	9	- ^d	-	9	-	-	0
10	2	11	-	-	7	2	2	0
	4	12	1	-	-	9	2	8
17	2	12	3	-	1	3	5	25
	4	12	6	1	1	3	1	50
	12	11	3	2	-	5	1	27
20	2	12	4 ^d	-	3	1	4	33
	4	11	5	1	-	2	3	45
	12	10	4	1	-	3	2	40

a. Uncultured allografts consisted of a whole foetal pancreas; a single foetal pancreas segment was used for cultured allografts.

b. Histological score key:

++++ Intact islets without mononuclear cell infiltration at the graft site.

+++ Intact islets with mononuclear cell infiltration at the graft site but no infiltration into islets.

++ Mononuclear cell infiltration and damaged islet tissue evident.

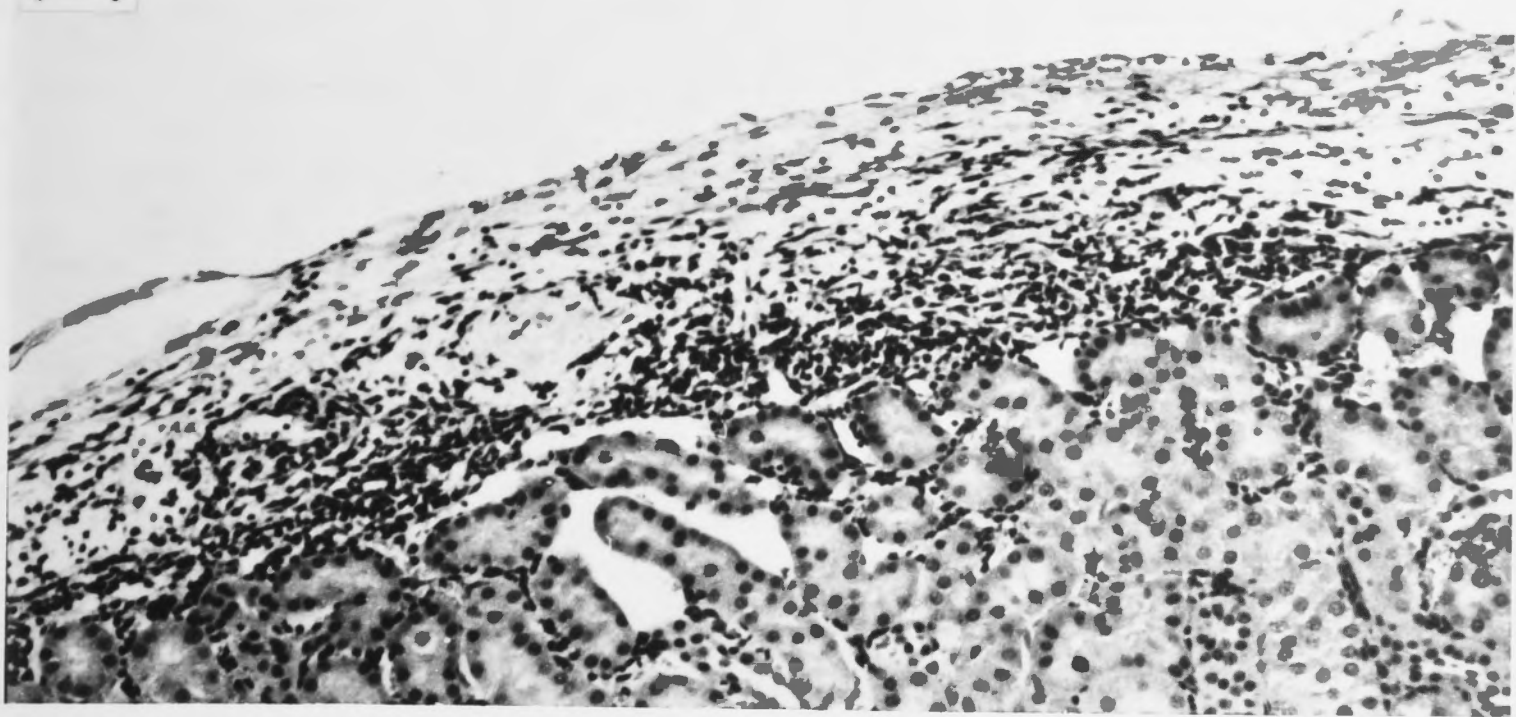
+ Mononuclear cell infiltration and scar tissue.

0 Fibrotic scar tissue or necrotic tissue without mononuclear cell infiltration.

c. Criteria for allograft survival were the presence of intact islets and the absence of mononuclear cell infiltration at the graft site.

d. $P = 0.1654$ (Exact probability test; Swinscow, 1980b).

(a)



(b)

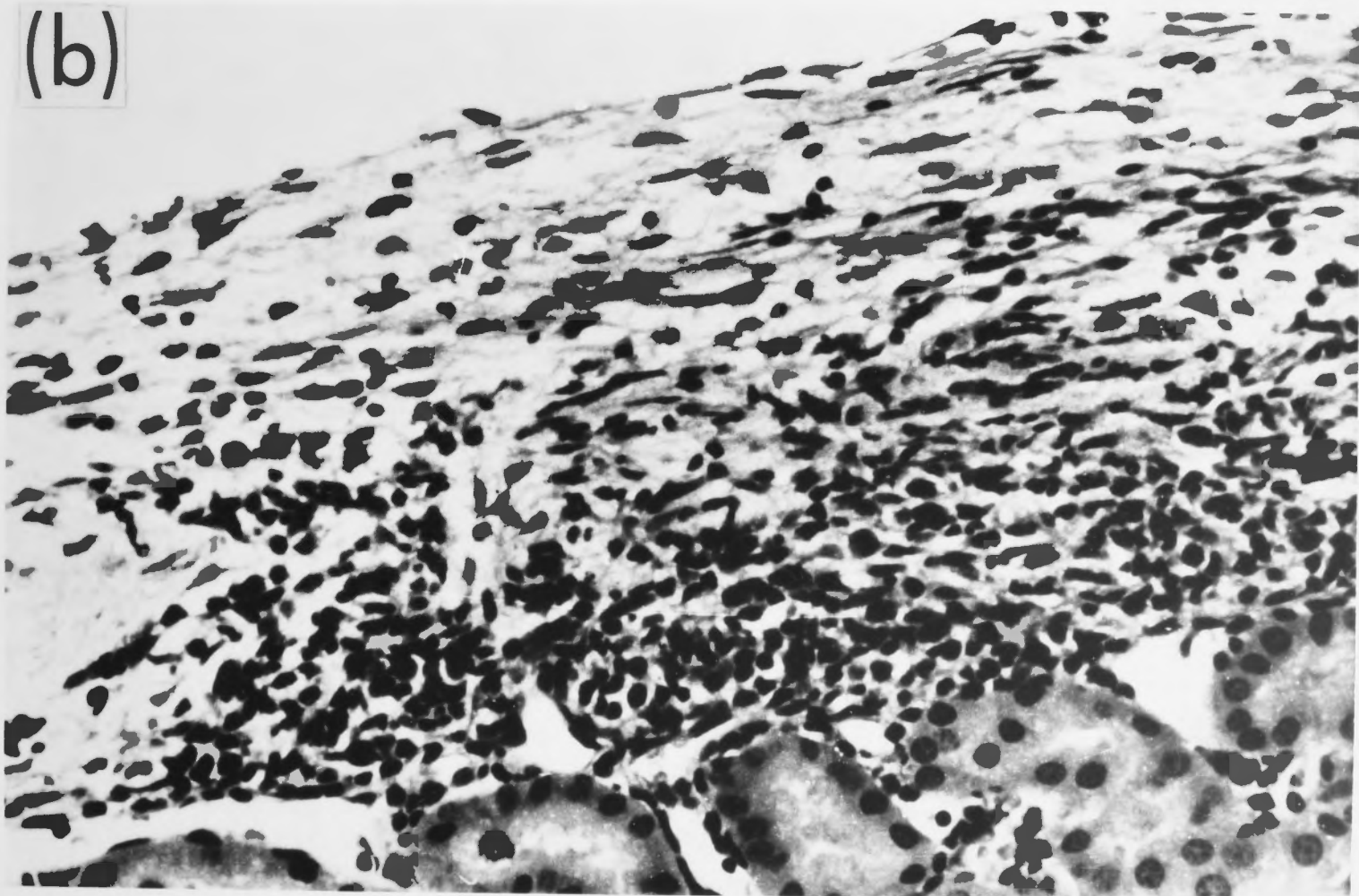


Figure 4.1 Allograft of uncultured BALB/c ($H-2^d$) foetal pancreas 2 weeks after transplantation under the kidney capsule of a CBA ($H-2^k$) recipient. Note the presence of scar tissue and mononuclear cell infiltration (a) Haematoxylin and eosin, x 167. (b) Higher magnification of the cell infiltrate shown in (a). Haematoxylin and eosin, x 407

transplantation were completely rejected. Histological examination revealed that seven of eleven 10 day-cultured allografts contained an intense mononuclear cell infiltrate and occasionally evidence of damaged islet tissue and duct epithelium (Fig. 4.2). At the macroscopic level, these grafts were markedly swollen in appearance. This finding was probably attributable to the vigorous inflammatory cell response. Two of eleven 10 day-cultured allografts at 2 weeks after transplantation consisted of scar tissue with mononuclear cell infiltration. The remaining two allografts were composed of fibrotic connective tissue and a few mononuclear cells. By 4 weeks after transplantation one of twelve 10 day-cultured allografts showed histological evidence of islet survival without mononuclear cell infiltration at the site of transplantation. However, nine of the twelve allografts showed obvious signs of rejection; these grafts contained scar tissue and a light mononuclear cell infiltrate. The remainder consisted of fibrotic connective tissue elements and a few mononuclear cells. In the case of those allografts which showed no evidence of islet survival or mononuclear cell infiltration, the histological appearance resembled scar tissue which repopulates a graft site following an acute allograft rejection reaction. This proposal is supported by the fact that all 10 day-cultured isografts showed islet development (see Chapter 3, Table 3.5). Even so, an alternative explanation is that the tissue was not viable at the time of the transplantation. Indeed, a small proportion of 10 day-cultured segments did not show histological survival of pancreatic tissue and predominantly

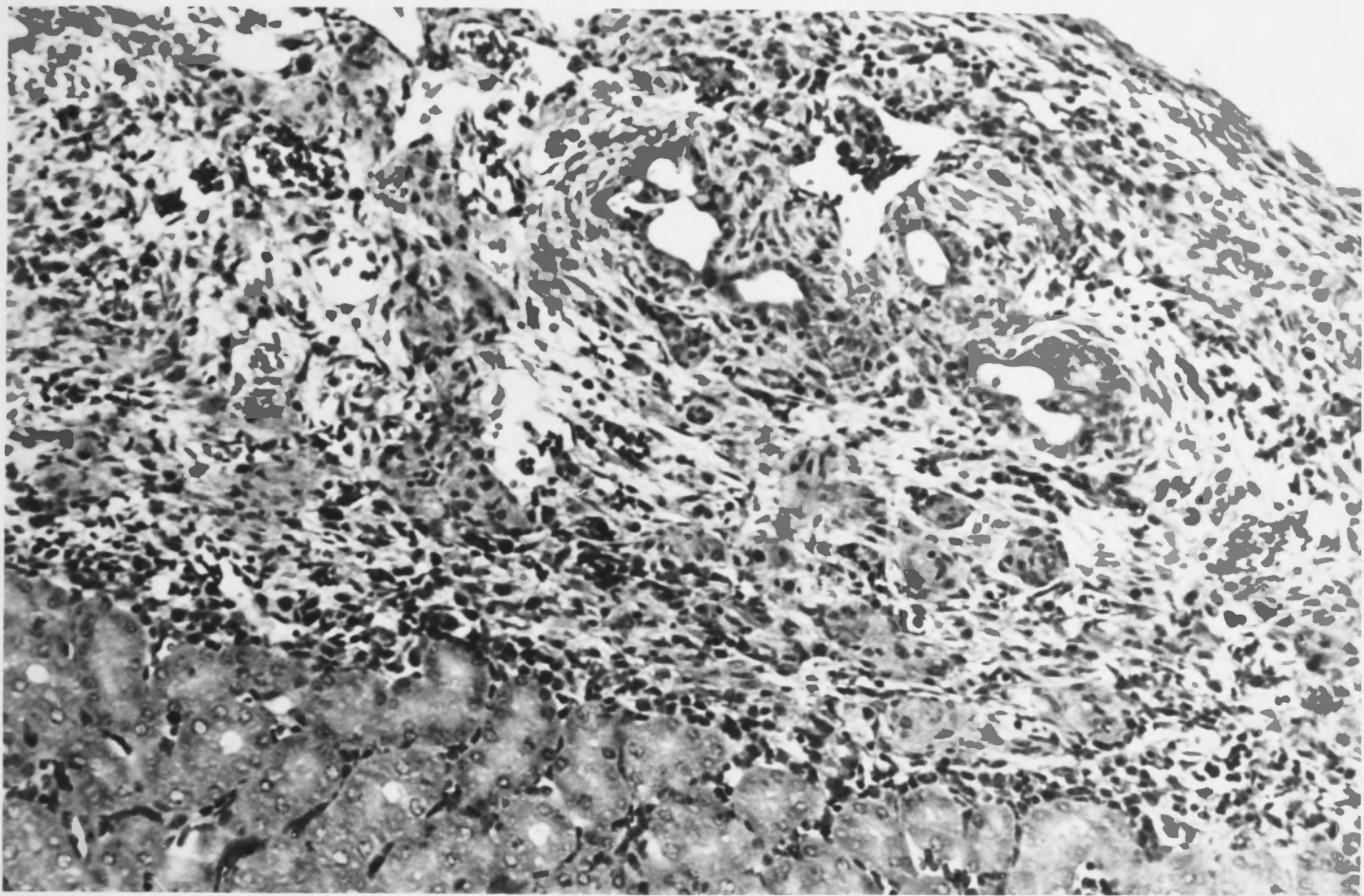
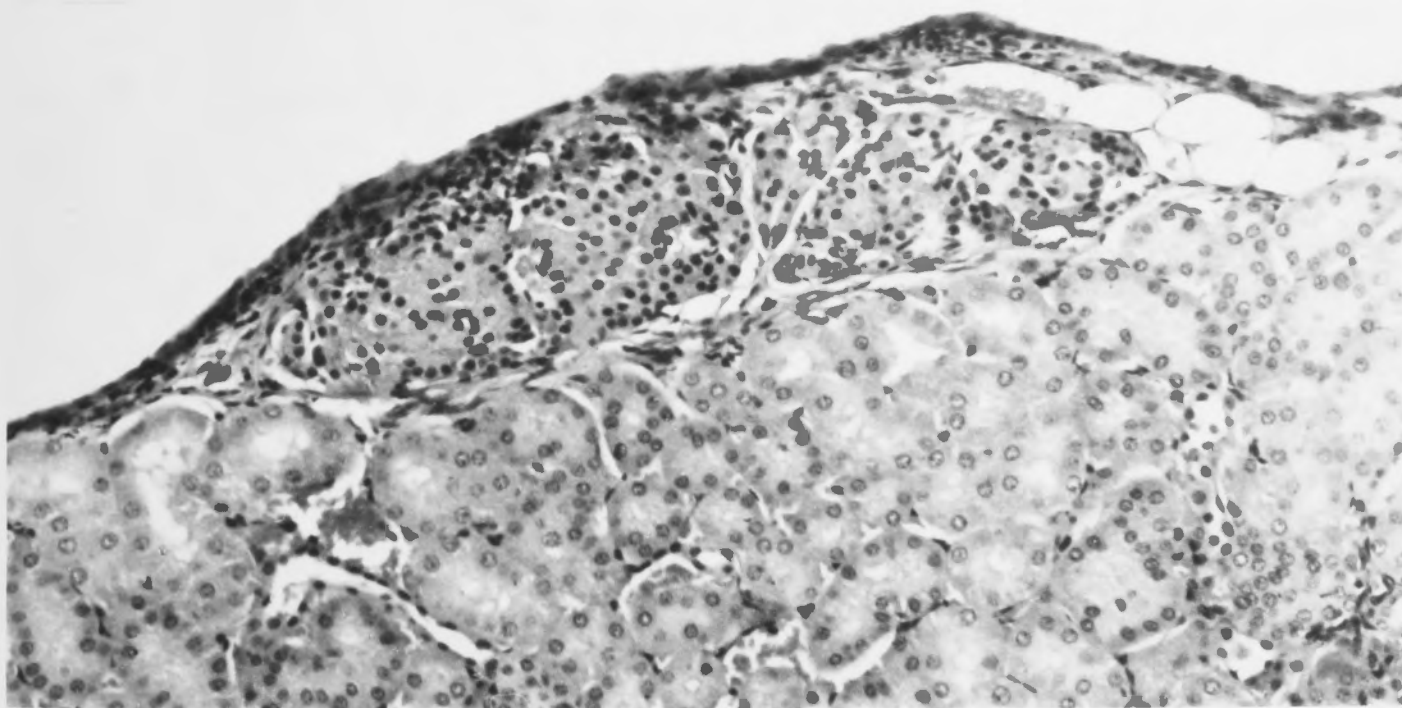


Figure 4.2 Allograft of a single 10 day-cultured BALB/c foetal pancreas segment 2 weeks after transplantation under the kidney capsule of a CBA recipient mouse. Note the mononuclear cell infiltration into graft. Haematoxylin and eosin, x 176.

consisted of fibrotic connective tissue (see Chapter 3, Table 3.4).

Evidence of allograft acceptance was substantially improved after prolonged organ culture for 17 to 20 days. However, there was no significant difference between the survival of allografts cultured for 17 and 20 days. Table 4.1 shows that, following 20 days in organ culture, four of twelve (33%), five of eleven (45%) and four of ten (40%) allografts, examined at 2, 4 and 12 weeks post-transplantation respectively, showed no evidence of rejection. These grafts consisted of well differentiated, intact islet tissue, duct epithelium, fibrotic connective tissue and some necrotic tissue (Fig. 4.3(a)). In addition, a small number of mononuclear cells, including histiocytes, were identified at the site of transplantation. These cell populations were also observed in isografts of cultured foetal pancreas tissue and were present either localized in a small region of the graft site or sparsely scattered in the connective tissue component of the graft. Therefore, the presence of these mononuclear cells was clearly distinguishable from the classical mononuclear cell infiltration reaction which characterizes the allograft rejection response. One allograft in each group examined at 4 and 12 weeks after transplantation contained intact islets and a mononuclear cell infiltrate which did not invade the islet tissue. In each group, 18-33% of the 20 day-cultured allografts showed obvious signs of rejection. These grafts showed mononuclear cell infiltration and sometimes damaged islet tissue or scar tissue (Fig. 4.3(b)). In addition, approximately 20-33% of the allografts

(a)



(b)

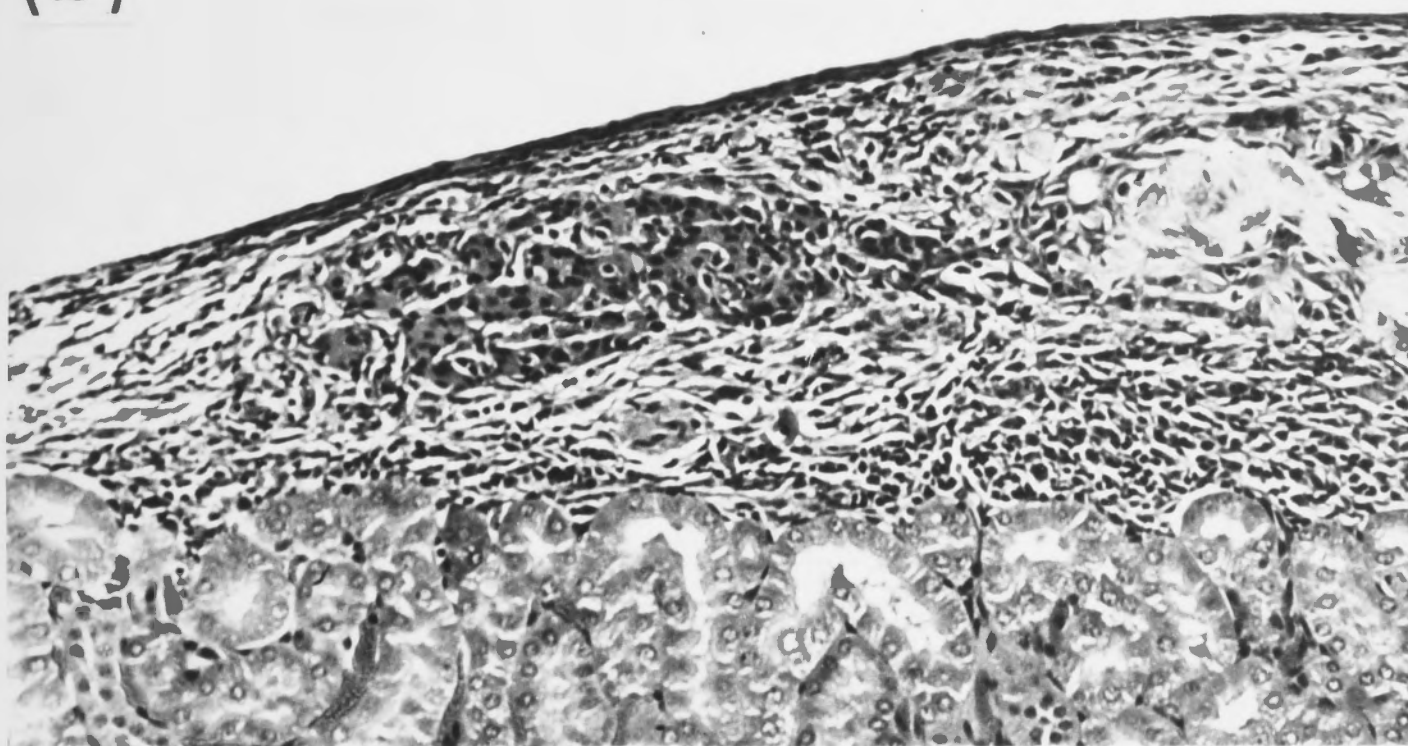


Figure 4.3 (a) Allograft of a single 20 day-cultured BALB/c foetal pancreas segment 4 weeks after transplantation under the kidney capsule of a CBA recipient mouse. Note the presence of intact, well developed islet tissue and absence of mononuclear cell infiltration. Haematoxylin and eosin, x 164. (b) Allograft of a 20 day-cultured BALB/c foetal pancreas segment, 2 weeks after transplantation beneath the kidney capsule of a CBA recipient. Note mononuclear cell infiltration into graft and damaged islet tissue. Haematoxylin and eosin, x 173.

in each group showed neither a rejection response nor significant islet development. The latter findings are consistent with the lack of islet development in a small proportion of 20 day-cultured isografts (see Chapter 3, Table 3.5). Thus, damage to foetal pancreas tissue during prolonged organ culture was probably responsible for the extensive fibrosis and necrosis observed in these 20 day-cultured allografts.

Although statistically there was no significant difference at the 5% level between the survival of uncultured and 20 day-cultured allografts (Table 4.1), there was a significant effect of organ culture on the histological appearance of foetal pancreas allografts. Seven of twelve 20 day-cultured allografts showed histological scores better than all nine uncultured allografts ($P = 0.0136$; Exact probability test; Swinscow, 1980b), at 2 weeks post-transplantation. It is possible that with larger group sizes, a significant effect of organ culture on allograft survival also would be observed.

This study indicates that prolonged organ culture in a 95% O_2 , 5% CO_2 gas phase for 17 to 20 days can facilitate the acceptance of 25-50% of foetal pancreas allografts. Organ culture for 20 days significantly improved the histological integrity of foetal pancreas allografts.

4.2.3 The effect of organ culture on the survival of adult islet allografts: a comparative study

In this study, we re-examined the effect of organ culture for 7 days in a high oxygen gas phase on the survival of allografts of isolated adult islets prepared from untreated

donor mice (Bowen *et al.*, 1980). Assessment of the integrity of cultured adult islet allografts was extended to 4 and 12 weeks post-transplantation. Graft survival was compared to cultured foetal pancreas allografts transplanted across the same major histocompatibility barrier.

Table 4.2 shows that all uncultured islet allografts at 2 and 4 weeks after transplantation contained a mononuclear cell infiltrate. Two of four and one of four grafts at 2 and 4 weeks post-transplantation respectively consisted of intact islet tissue and a mononuclear cell infiltrate which surrounded but did not penetrate the islet tissue. In both groups, one of four grafts showed heavy mononuclear cell infiltration and obvious destruction of islet architecture. The remaining allografts were completely rejected; there was heavy mononuclear cell infiltration and only remnants of islet tissue were evident (Fig. 4.4(a)).

The survival of cultured islet allografts at 4 weeks after transplantation was significantly better than for corresponding uncultured islet allografts ($P = 0.0284$). Three of four (75%), four of four (100%) and five of five (100%) cultured allografts, at 2, 4 and 12 weeks post-transplantation respectively, showed no signs of rejection. The grafts were composed of intact islet tissue which stained intensely with aldehyde fuchsin (Fig. 4.4(b)). The remaining allograft at 2 weeks after transplantation contained intact islet tissue and a light mononuclear cell infiltrate which did not invade the islet mass.

Table 4.3 shows that when 10^5 viable donor strain peritoneal cells were injected into recipient mice at the time of allotransplantation of 7 day-cultured adult islets, all of five

TABLE 4.2

Effect of organ culture on the survival of allografts of
isolated adult islets^a

Duration of culture (days)	Time post- transplant (weeks)	Total No. grafts examined	No. grafts with histological score ^b					% grafts surviving ^c
			++++	+++	++	+	0	
0	2	4	-	2	1	1	-	0
	4	4	- ^d	1	1	2	-	0
7	2	4	3	1	-	-	-	75
	4	4	4 ^d	-	-	-	-	100
	12	5	5	-	-	-	-	100

a. Allografts consisted of a single cluster of isolated adult islets (Section 2.6; Section 2.11).

b. Histological score key:

++++ Intact islet tissue without mononuclear cell infiltration at the graft site.

+++ Intact islet tissue with mononuclear cell infiltration at the graft site but no infiltrate in islet tissue.

++ Mononuclear cell infiltration and damaged islet tissue evident.

+ Mononuclear cell infiltration and scar tissue.

0 Fibrotic scar tissue or necrotic tissue without mononuclear cell infiltration.

c. Criteria for allograft survival were the presence of intact islet tissue without mononuclear cell infiltration at the graft site.

d. $P = 0.0284$ (Exact probability test; Swinscow, 1980b).

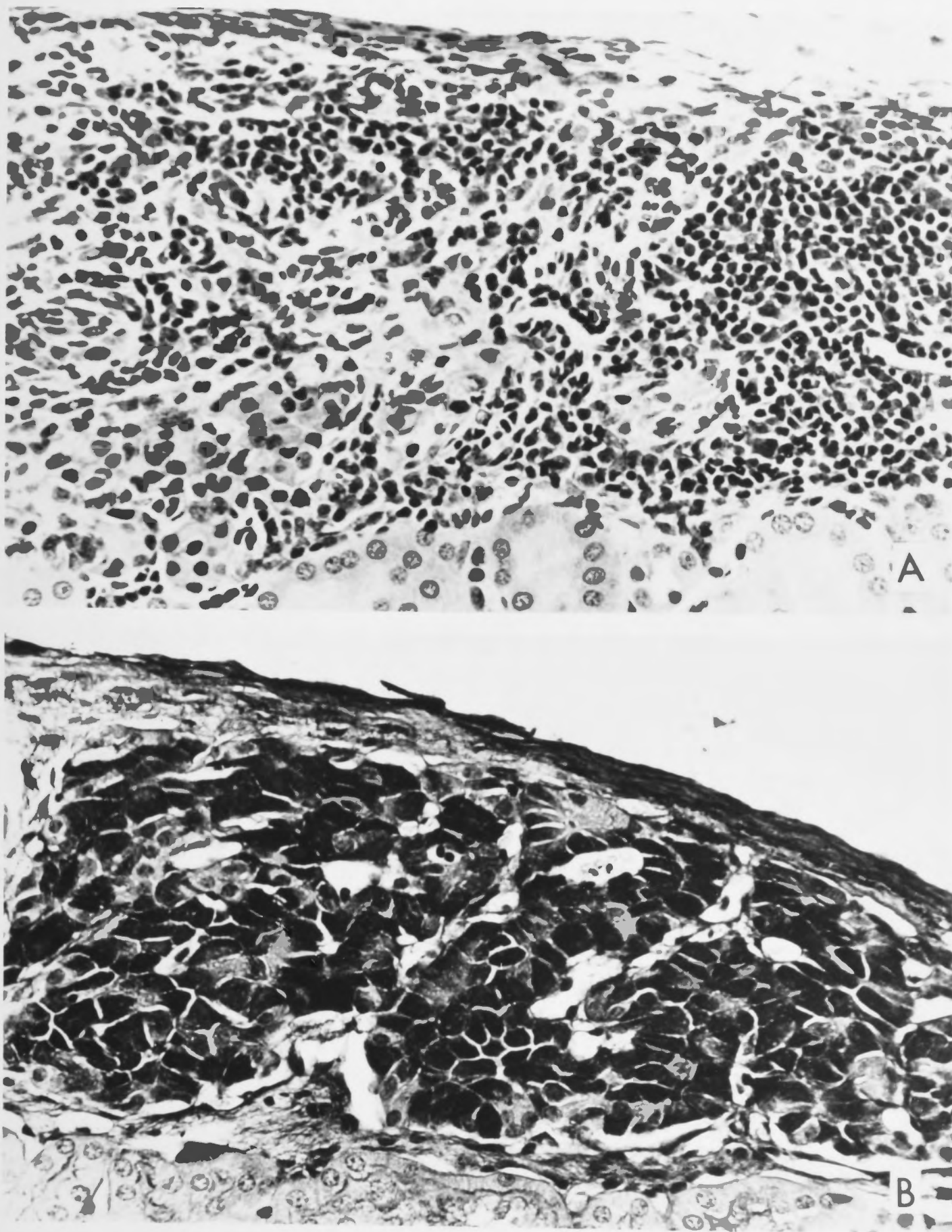


Figure 4.4 A. Allograft response to BALB/c ($H-2^d$) isolated adult islets 2 weeks after transplantation under the kidney capsule of a CBA ($H-2^k$) recipient mouse. Note heavy mononuclear cell infiltration into graft. Haematoxylin and eosin, x 354. B. Allograft of 7 day-cultured BALB/c isolated adult islets 12 weeks post-transplantation under the kidney capsule of a CBA recipient. Note dark staining β cells present in the transplanted tissue and lack of any allograft response. Aldehyde fuchsin, x 316.

TABLE 4.3

The effect of an intravenous injection of viable peritoneal cells (PC) to CBA mice at the time of allotransplantation of 7 day-cultured BALB/c adult islets.

Cell injection	Total No. mice	No. mice with surviving allografts ^{a,b,c}	% allografts surviving
10^5 BALB/c PC	5	0	0
10^5 CBA PC	5	5	100

- a. Allografts were examined histologically at 2 weeks after transplantation.
- b. Criteria for allograft survival were the presence of intact islet tissue and the absence of mononuclear cell infiltration at the graft site.
- c. Allografts consisted of a 7 day-cultured cluster of isolated adult islets (Section 2.6).

cultured allografts were rapidly rejected. In all of five control mice, which received transplants of the same cultured islet tissue, the injection of recipient type peritoneal cells had no effect on allograft survival. This finding demonstrated that cultured adult islet tissue carries recognizable antigens which can act as targets following active immunization of the transplant recipients with donor-specific stimulator cells (Sections 1.5.3, 1.6.1).

Thus, organ culture alone for 7 days is sufficient pre-treatment to obtain long-term survival of up to 100% of adult islet allografts. In contrast, organ culture for 17 to 20 days in the same gas phase is necessary to show evidence of acceptance of 25-50% of foetal pancreas allografts, using the same donor and recipient mouse strains (Section 4.2.2). This study clearly demonstrates that organ culture is more effective in reducing the immunogenicity of adult islet tissue than in the case of the foetal pancreas. Furthermore, the effect of organ culture on the survival of adult islet allografts is not due to the loss of tissue antigenicity; the cultured adult islets were shown to carry recognizable antigens.

4.2.4 The effect of organ culture on the survival of allografts and isografts of six foetal pancreas segments

It is evident from the data presented in this chapter (Section 4.2.2) that prolonged organ culture for 17 to 20 days is required to obtain acceptance of a proportion of foetal pancreas allografts consisting of a single foetal pancreas

segment. However, it is apparent from the studies of isograft function, detailed in Chapter 3 (Section 3.2.6), that at least six 20 day-cultured foetal pancreas segments (tissue derived from two donor foetal pancreases) are required to reverse diabetes in isogeneic recipient mice. Thus, to determine the optimal culture conditions to be used for a study of allograft function (Section 4.2.6) we re-investigated the effect of organ culture on prolonging foetal pancreas allograft survival. On this occasion, however, each allograft consisted of six foetal pancreas segments. BALB/c segments were cultured for 10, 17, 20 and 24 days in a 95% O₂, 5% CO₂ gas phase prior to allotransplantation to CBA recipient mice. The culture duration was extended to 24 days in an attempt to improve allograft survival (Section 4.2.2).

Table 4.4 shows that all allografts of six 10 day-cultured foetal pancreas segments were acutely rejected and, in general, resembled the histological appearance of single 10 day-cultured segment allografts (Section 4.2.2). As for single segment allografts, evidence of acceptance of allografts of six foetal pancreas segments was observed after 17 to 20 days of organ culture. There was no significant difference between the survival of six segment allografts cultured for 17 and 20 days. Although there is an apparent trend for the survival of allografts consisting of six 17 day-cultured and six 20 day-cultured segments to be lower than for corresponding single segment allografts (Table 4.1; Table 4.4), this difference was not statistically significant at the 5% level.

In Table 4.4, three of ten and two of eight 20 day-cultured allografts showed no evidence of rejection at 4 and

TABLE 4.4

Survival of allografts consisting of six cultured foetal
pancreas segments

Duration of culture (days)	Time post- transplant (weeks)	Total No. grafts examined	No. grafts with histological score ^a					% grafts surviving ^b
			++++	+++	++	+	0	
10	2	16	-	-	5	11	-	0
17	2	10	1	-	8	1	-	10
	4	10	2	-	3	3	2	20
20	2	8	- ^{c, d}	2	5	1	-	0
	4	10	3 ^c	-	3	4	-	30
	12	8	2 ^d	2	-	1	3	25
24	2	9	-	2	1	1	5	0
	4	10	2	1	3	2	2	20

a. Histological score key:

++++ Intact islets without mononuclear cell infiltration at the graft site.

+++ Intact islets with mononuclear cell infiltration at the graft site but no infiltration into islets.

++ Mononuclear cell infiltration and damaged islet tissue present.

+ Mononuclear cell infiltration and scar tissue.

0 Fibrotic scar tissue or necrotic tissue without mononuclear cell infiltration.

b. Criteria for allograft survival were the presence of intact islets and the absence of mononuclear cell infiltration at the graft site.

c. $P = 0.2942$ (Exact probability test; Swinscow, 1980b).

d. $P = 0.4666$ (Exact probability test; Swinscow, 1980b).

12 weeks post-transplantation respectively. These grafts contained intact, aldehyde fuchsin-positive islets, some duct epithelium, connective tissue and occasionally a few mononuclear cells (Fig. 4.5). There was no evidence of mononuclear cell infiltration. Compared to the proportion of allografts surviving at 4 and 12 weeks, the lack of allograft survival in the group examined at two weeks post-transplantation was not statistically significant at the 5% level ($P = 0.2942$; $P = 0.4666$, respectively). It should be noted that two of eight grafts in this same group, and also at 12 weeks post-transplantation, contained intact islets and a mononuclear cell infiltrate which did not invade the islet tissue (Fig. 4.6). It is quite possible that such an infiltrate could eventually dissipate without jeopardizing islet integrity. Except for three of eight grafts at 12 weeks post-transplantation, which were mainly composed of fibrotic connective tissue and a small number of mononuclear cells, the remaining grafts showed obvious signs of rejection.

No advantage was gained by extending the culture period to 24 days. In terms of allograft survival, there was no significant difference between 20 day-cultured and 24 day-cultured allografts.

Table 4.5 shows that following organ culture for up to 17 days, all isografts of six foetal pancreas segments contained well differentiated islets. After 20 days of organ culture one of seventeen isografts showed poor islet development; this graft was composed mainly of connective tissue and contained some remnants of islet tissue and some necrotic tissue. The majority of 20 day-cultured isografts contained

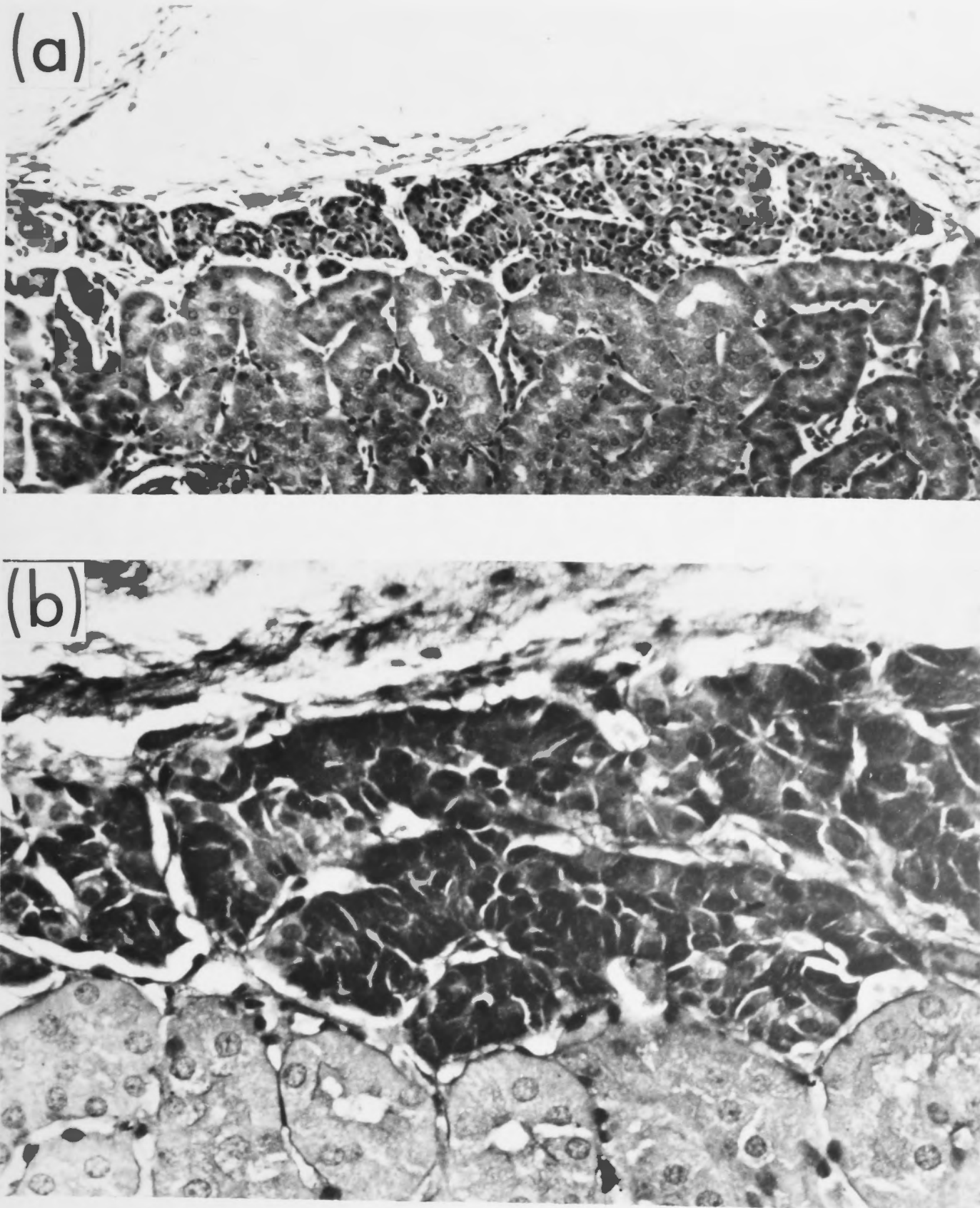


Figure 4.5 (a) Allograft of six 20 day-cultured BALB/c foetal pancreas segments 12 weeks after transplantation under the kidney capsule of a CBA recipient mouse. Note well differentiated islet tissue and absence of mononuclear cell infiltration into graft. Haematoxylin and eosin, x 162. (b) Higher magnification of the cultured allograft in (a), showing presence of densely granulated beta cells. Aldehyde fuchsin, x 405.

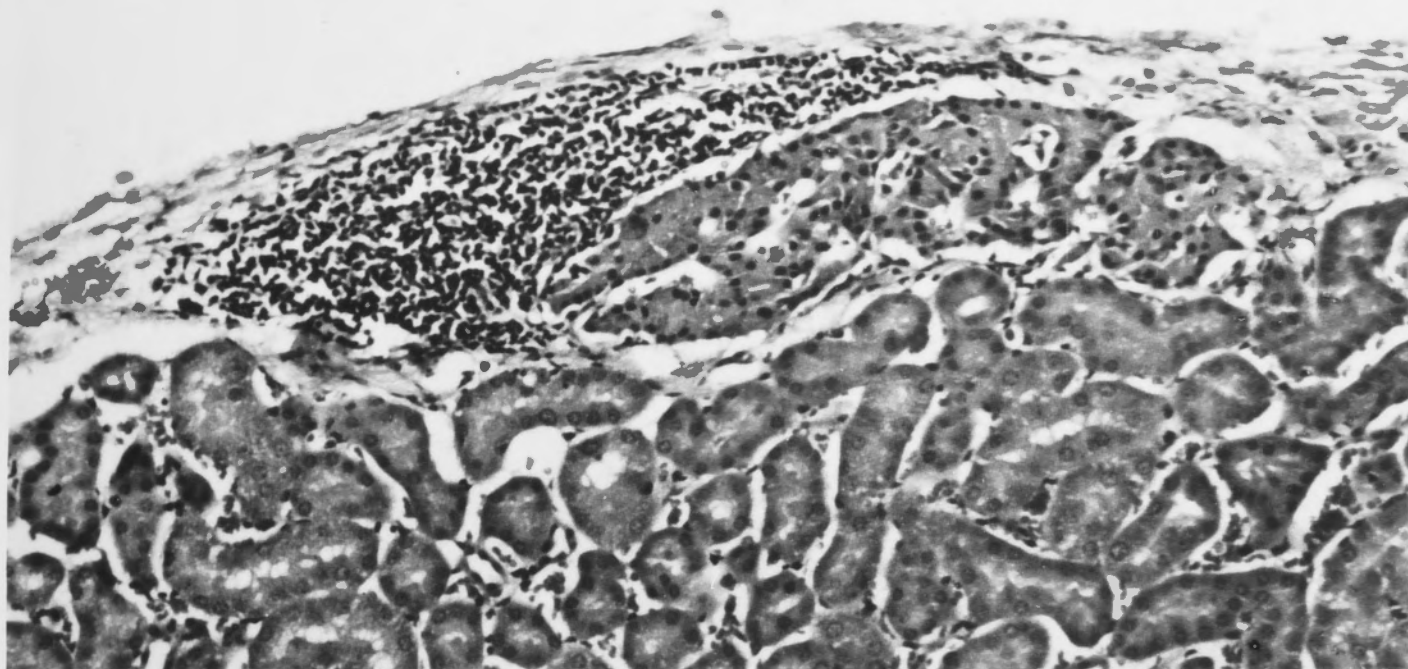


Figure 4.6 Allograft of six 20 day-cultured BALB/c foetal pancreas segments at 12 weeks post-transplantation beneath the kidney capsule of a CBA recipient. Note intact islet tissue and the presence of mononuclear cells which do not invade the islet tissue. Haematoxylin and eosin, x 167.

TABLE 4.5

Islet development in isografts of six cultured foetal pancreas segments

Time in culture (days)	No. transplants examined ^a	No. transplants showing well developed islets	No. transplants with poor islet development ^b	% transplants showing islet survival
10	6	6	-	100
17	20	20	- ^c	100
20	17	16	1	94
24	13	8	5 ^c	61

a. Tissue examined 2-4 weeks after transplantation.

b. Transplants consisted mainly of fibrotic connective tissue with remnants of islet tissue.

c. $P = 0.0108$ (Exact probability test; Swinscow, 1980b).

well developed, aldehyde fuchsin-positive islets. Compared to 17 day-cultured isografts, a significant decline in islet survival was seen in isografts consisting of six 24 day-cultured segments ($P = 0.0108$). Whereas all of twenty 17 day-cultured isografts contained well developed islet tissue, five of thirteen 24 day-cultured isografts contained only remnants of islet tissue. These findings support the proposal that prolonged organ culture in a 95% O_2 , 5% CO_2 gas phase damages foetal islet tissue (Section 3.2.5). Furthermore, like isografts of cultured single segments, isografts of six cultured foetal pancreas segments did not contain lymph node structures (Section 3.2.5).

Thus, in terms of both improvement in allograft integrity and preservation of islet development, 20 days represents the maximum duration of organ culture for foetal mouse pancreas tissue. At 12 weeks after transplantation, 25% of allografts consisting of six 20 day-cultured foetal pancreas segments showed no evidence of rejection.

4.2.5 Function of uncultured foetal pancreas allografts

In view of the decision to use tissue from two donor foetal pancreases to investigate the function of cultured foetal pancreas allografts (Section 4.2.4), we examined first the functional capacity of allografts consisting of two uncultured foetal pancreases to reverse Streptozotocin-induced diabetes.

Figure 4.7 shows the blood glucose response of diabetic CBA mice following allotransplantation of two uncultured BALB/c foetal pancreases under the kidney capsule. The data

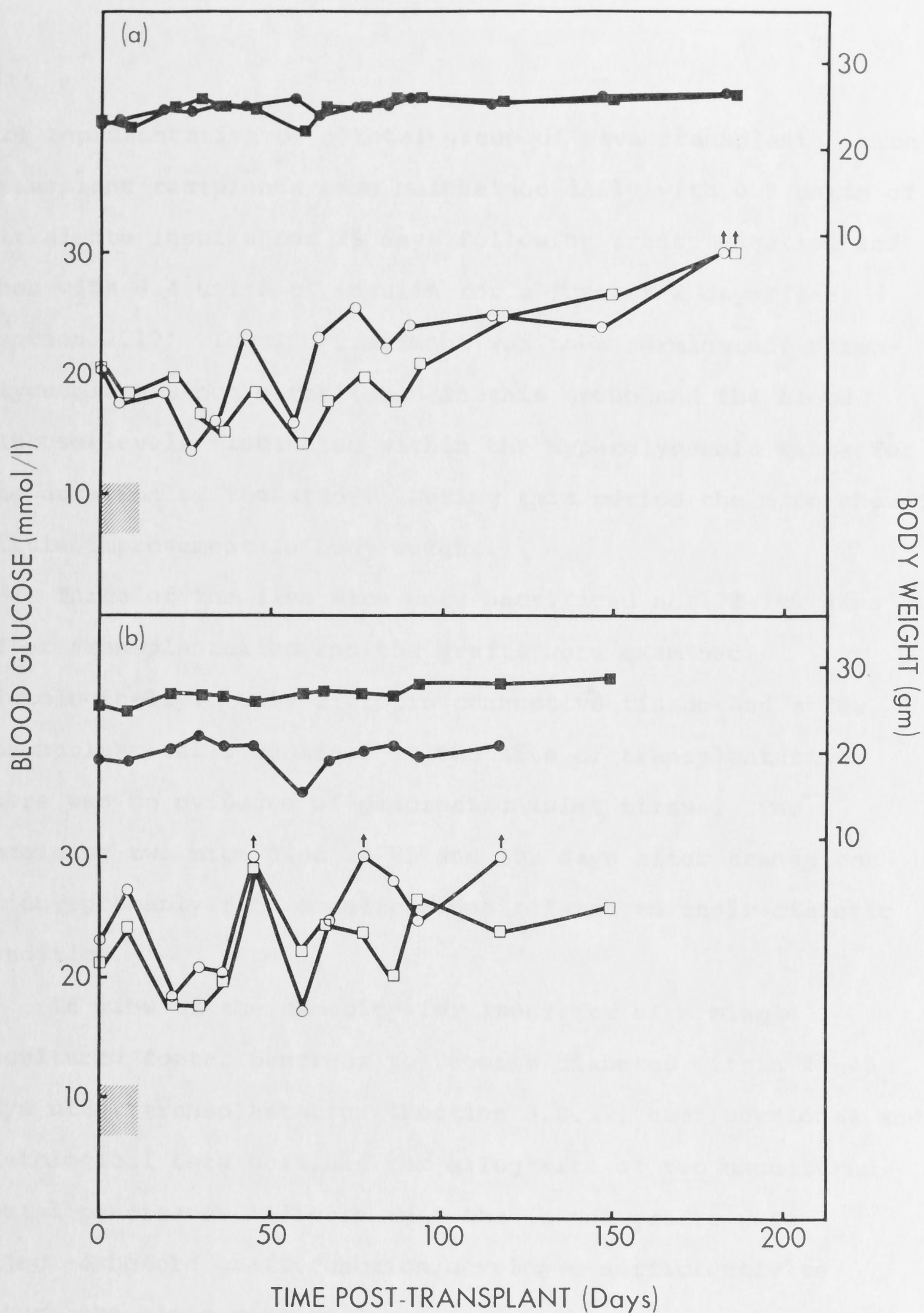


Figure 4.7 Non-fasting blood glucose levels (open symbols) and body weight (closed symbols) of diabetic CBA mice following allotransplantation of two uncultured BALB/c foetal pancreases beneath the kidney capsule. The shaded region represents the 95% probability interval for the non-fasting blood glucose levels of normal CBA mice. Each panel shows the data for two transplanted mice.

are representative of a total group of five transplanted mice. Transplant recipients were maintained daily with 0.8 units of Ultralente insulin for 29 days following transplantation and then with 0.4 units of insulin for a further 2 days (see Section 2.17). Insulin treatment was then terminated. Normoglycaemia was not established in this group and the blood glucose levels fluctuated within the hyperglycaemic range for the duration of the study. During this period the mice showed little improvement in body weight.

Three of the five mice were sacrificed at 122-186 days after transplantation and the grafts were examined histologically. Only fibrotic connective tissue and a few mononuclear cells remained at the site of transplantation. There was no evidence of pancreatic islet tissue. The remaining two mice died at 85 and 179 days after transplantation, probably from complications related to their diabetic condition.

In view of the capacity for isografts of a single uncultured foetal pancreas to reverse diabetes within 23-45 days after transplantation (Section 3.2.3), the functional and histological data obtained for allografts of two uncultured foetal pancreases indicate that the latter grafts were rejected before graft function developed sufficiently to return the blood glucose levels to within the normal range.

4.2.6 Function of 20 day-cultured foetal pancreas allografts

On the basis of the histological data presented in Section 4.2.4 and the failure of uncultured allografts to reverse diabetes (Section 4.2.5), we investigated the

functional capacity of 20 day-cultured allografts to ameliorate Streptozotocin-induced diabetes in CBA recipient mice.

A group of seven diabetic CBA mice received transplants of six 20 day-cultured BALB/c foetal pancreas segments under the kidney capsule. Figure 4.8(a) shows that normoglycaemia was restored in two of the seven transplant recipients within 29-37 days after transplantation. These two mice were maintained with 0.8 units of Ultralente insulin for 31 days after transplantation. The non-fasting blood glucose level of another transplanted mouse was returned to within the normal range by 101 days after transplantation. This mouse was maintained with 0.8 units of Ultralente insulin for 90 days after transplantation. However, the blood glucose level fluctuated out of the normal range until 173 days after transplantation. Thereafter, the normoglycaemia remained stable. In each of these three transplant recipients, graft function was accompanied by a dramatic increase in body weight and the re-establishment of aglycosuria (Table 4.6). Following surgical removal of the graft, each mouse rapidly became diabetic. Thus, allograft function alone had been responsible for normalizing the glucose metabolism in these mice. Histological examination of these allografts showed the presence of intact islet tissue without any evidence of mononuclear cell infiltration (Fig. 4.9).

Figure 4.8(b) shows the data for two of the remaining four allograft recipients which failed to demonstrate successful reversal of diabetes. All four transplanted mice were maintained with 0.8 units of Ultralente insulin for 115-122 days after transplantation. The data presented in Figure

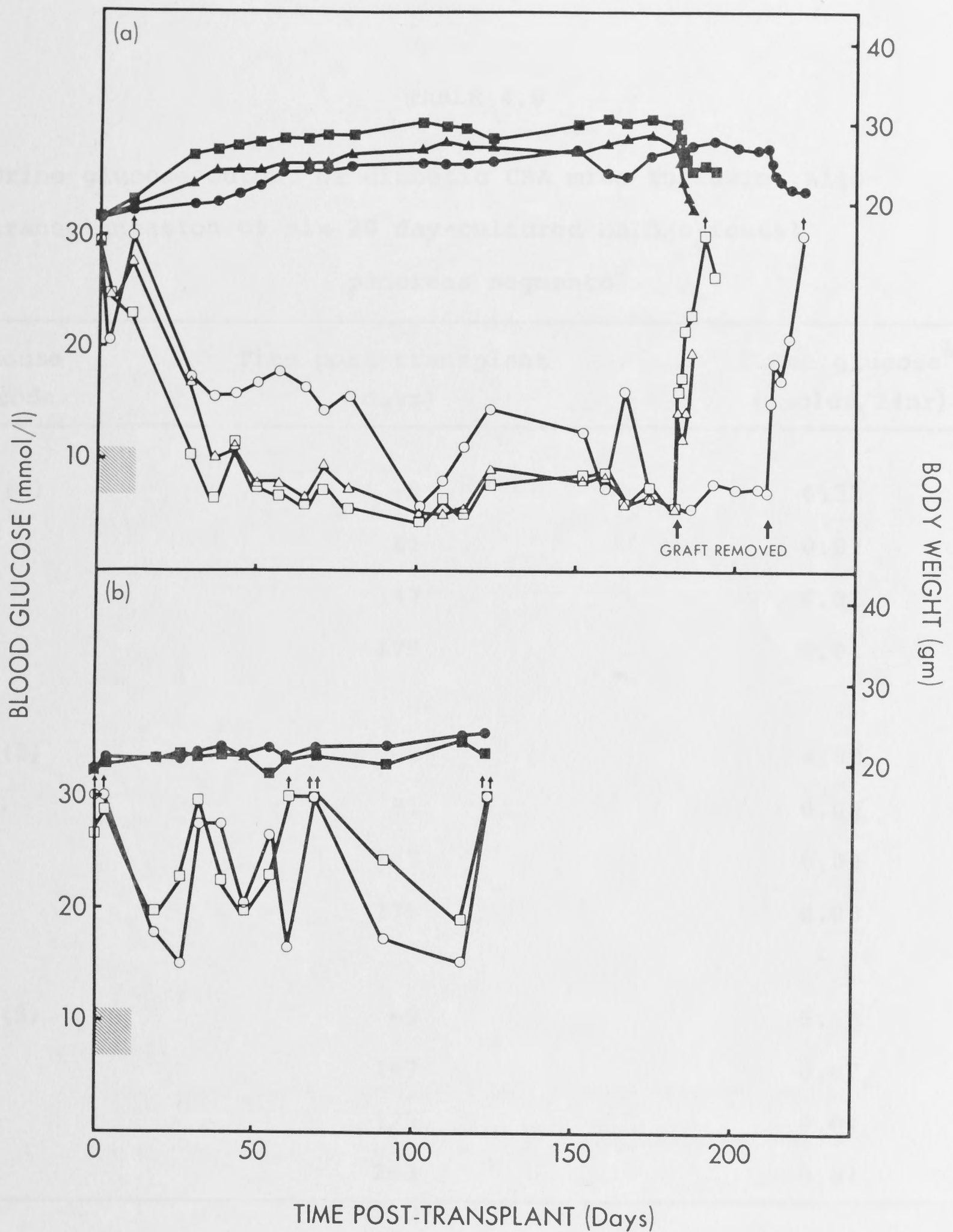


Figure 4.8 Non-fasting blood glucose levels (open symbols) and body weight (closed symbols) of diabetic CBA mice following allotransplantation of six 20 day-cultured BALB/c foetal pancreas segments under the kidney capsule. The shaded region defines the 95% probability interval for the non-fasting blood glucose levels of normal CBA mice. Panels (a) and (b) show the data for three and two transplant recipients respectively. Grafts (a) were removed by excision of the graft-bearing kidney.

TABLE 4.6

Urine glucose output of diabetic CBA mice following allo-transplantation of six 20 day-cultured BALB/c foetal pancreas segments^a.

Mouse code	Time post-transplant (days)	Urine glucose ^b (mmoles/24hr)
(1)	-5	6.32
	81	0.07
	147	0.01
	175	0.01
(2)	-5	4.88
	81	0.09
	147	0.00
	175	0.00
(3)	-5	5.55
	147	0.07
	175	0.04
	203	0.01

a. The table shows the data for the three transplant recipients in which normoglycaemia was re-established.

b. Urine glucose output for normal CBA mice was ≤ 0.04 mmoles glucose/24 hours (Section 2.18).

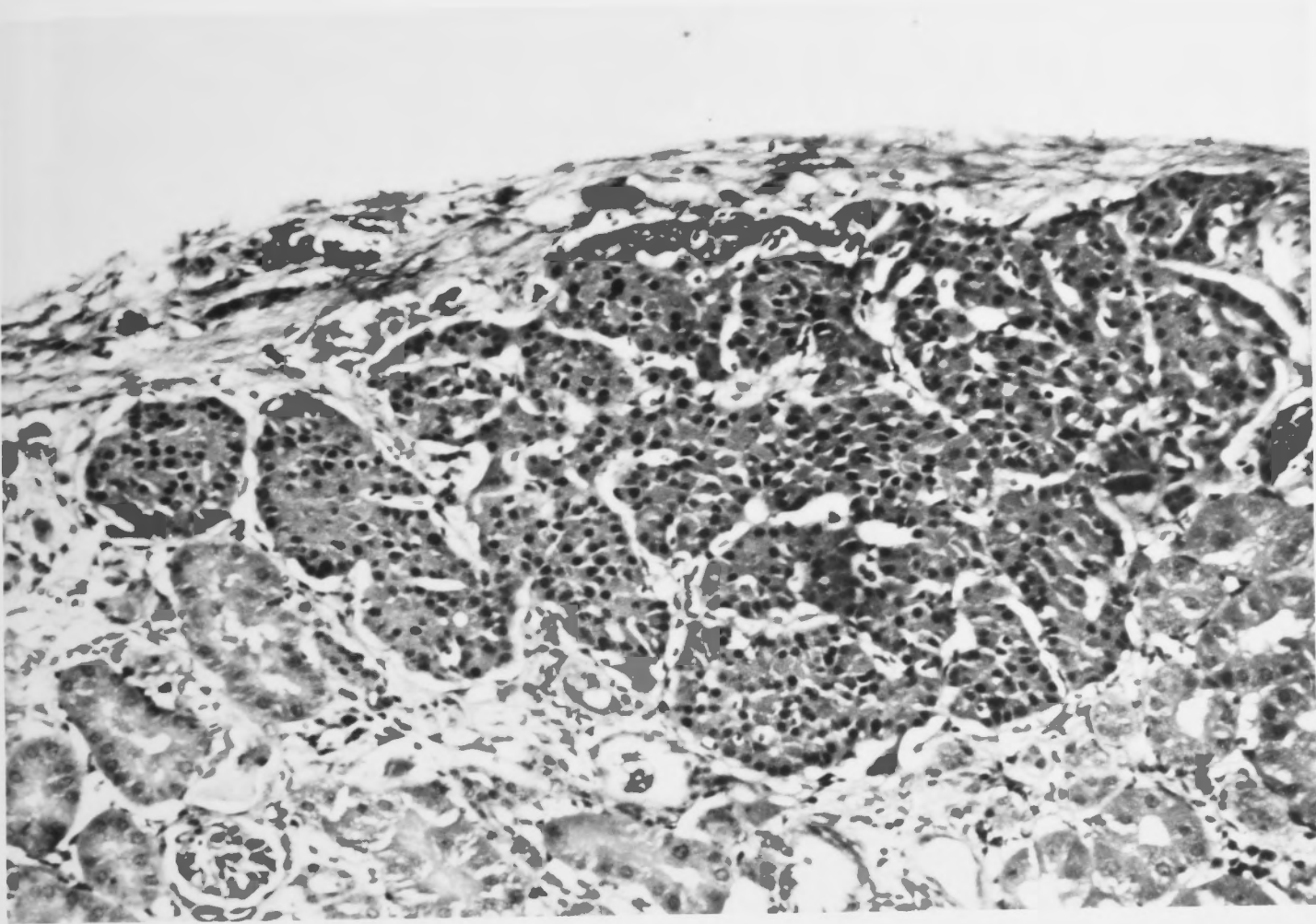


Figure 4.9 Allograft of six 20 day-cultured BALB/c foetal pancreas segments at 181 days after transplantation under the kidney capsule of a diabetic CBA recipient mouse. Note well differentiated islet tissue and absence of mononuclear cell infiltration. Haematoxylin and eosin, x 171.

4.8(b) suggest that a temporary improvement in the hyperglycaemic state may have occurred up to 27 days after transplantation. Thereafter, the blood glucose levels showed marked fluctuations within the diabetic range. These two transplant recipients were sacrificed at 120 days after transplantation and the integrity of the allografts was assessed. At the histological level, one of the two grafts showed well differentiated, intact islet tissue and some mononuclear cell infiltration which did not invade the islets. It is possible therefore that endocrine function may have continued to develop if this graft had remained *in situ*. The other graft showed scar tissue, a few mononuclear cells and no evidence of islet tissue. The remaining two mice died at 120 and 127 days after transplantation without having recovered from their diabetic condition during the preceding week.

Thus, 43% of allografts consisting of six 20 day-cultured foetal pancreas segments (20 day-cultured tissue derived from two donor foetal pancreases) were successful in reversing Streptozotocin-induced diabetes. This finding is consistent with the histological survival of 20 day-cultured allografts (Section 4.2.4). Interestingly, 20 day-cultured allografts functioned much more efficiently than 20 day-cultured isografts (Section 3.2.6). We suggest that this difference is probably attributable to the development of our technical expertise in maintaining foetal pancreas tissue in organ culture.

4.2.7 The effect of organ culture and ALS treatment of transplant recipients on the survival of foetal pancreas allografts

In this chapter we have demonstrated partial success in facilitating foetal pancreas allograft acceptance by organ culture (Section 4.2.2; Section 4.2.4). In relation to allografts of isolated adult islets, Lacy and co-workers (1979a) reported improved survival of cultured rat islets following administration of a single dose of anti-lymphocyte serum (ALS) to transplant recipients at the time of transplantation. In view of this success, we investigated the effect of short-term ALS treatment of transplant recipients on the survival of cultured and uncultured foetal pancreas allografts (see Section 2.25).

Table 4.7 shows the survival of BALB/c foetal pancreas allografts following organ culture for 0, 10 and 20 days, and transplantation to either ALS-treated or untreated CBA recipient mice. In the case of uncultured foetal pancreas allografts, ALS-treatment marginally delayed graft rejection. Uncultured allografts from control mice were completely rejected by 2 weeks post-transplantation. At 4 weeks post-transplantation, one of four grafts in the ALS-treated group contained intact islet tissue which did not show mononuclear cell infiltration. The remaining grafts showed obvious signs of rejection. All of four grafts examined at 8 weeks post-transplantation showed evidence of rejection.

The survival of 10 day-cultured allografts was considerably prolonged following ALS-treatment of recipient mice. All 10 day-cultured allografts from untreated

TABLE 4.7

The effect of ALS treatment of transplant recipients on islet survival in foetal pancreas allografts^a

Duration of culture (days)	Recipient treatment	Time post-transplant (weeks)	Total No. grafts examined	No. grafts showing islet survival ^b	% grafts showing islet survival
0	Normal	2	3	0	0
	ALS	4	4	1	25
		8	4	0	0
10	Normal	2	16	0	0
	ALS	4	4	4	100
		8	4	0	0
20	Normal	8	5	4	80
	ALS	8	8	7	88

a. Uncultured allografts consisted of two whole foetal pancreases; cultured allografts were composed of six foetal pancreas segments.

b. Criteria for islet survival were the presence of intact islet tissue which showed no evidence of mononuclear cell infiltration.

recipients showed obvious signs of rejection by 2 weeks after transplantation (Fig. 4.10(a)). In contrast, at 4 weeks post-transplantation, all of four 10 day-cultured allografts in the ALS-treated group contained intact islet tissue which showed no evidence of mononuclear cell infiltration (Fig. 4.10(b)). These grafts also contained duct epithelium and some adipose tissue. However, by 8 weeks after transplantation graft rejection was evident and no intact islet tissue remained.

In terms of improved islet survival, no effect of ALS was demonstrated in 20 day-cultured allografts examined at 8 weeks after transplantation.

A statistical analysis of these data was precluded on the basis that uncultured and 10 day-cultured allografts in normal recipient mice were assessed for islet survival at an earlier post-transplant time than for the ALS-treated groups. However since the former grafts showed evidence of acute rejection, the data indicate that short-term immunosuppression of transplant recipients with ALS delayed but did not prevent the rejection of 10 day-cultured foetal pancreas allografts. The study suggests that organ culture of foetal pancreas tissue for 10 days in a high oxygen gas phase and ALS treatment of transplant recipients acted synergistically to produce a transient prolongation in the survival of foetal pancreas allografts.

4.3 DISCUSSION

It is evident from the data presented in this chapter that organ culture for 20 days in a high oxygen atmosphere can significantly improve the histological integrity of foetal pancreas allografts transplanted across a major

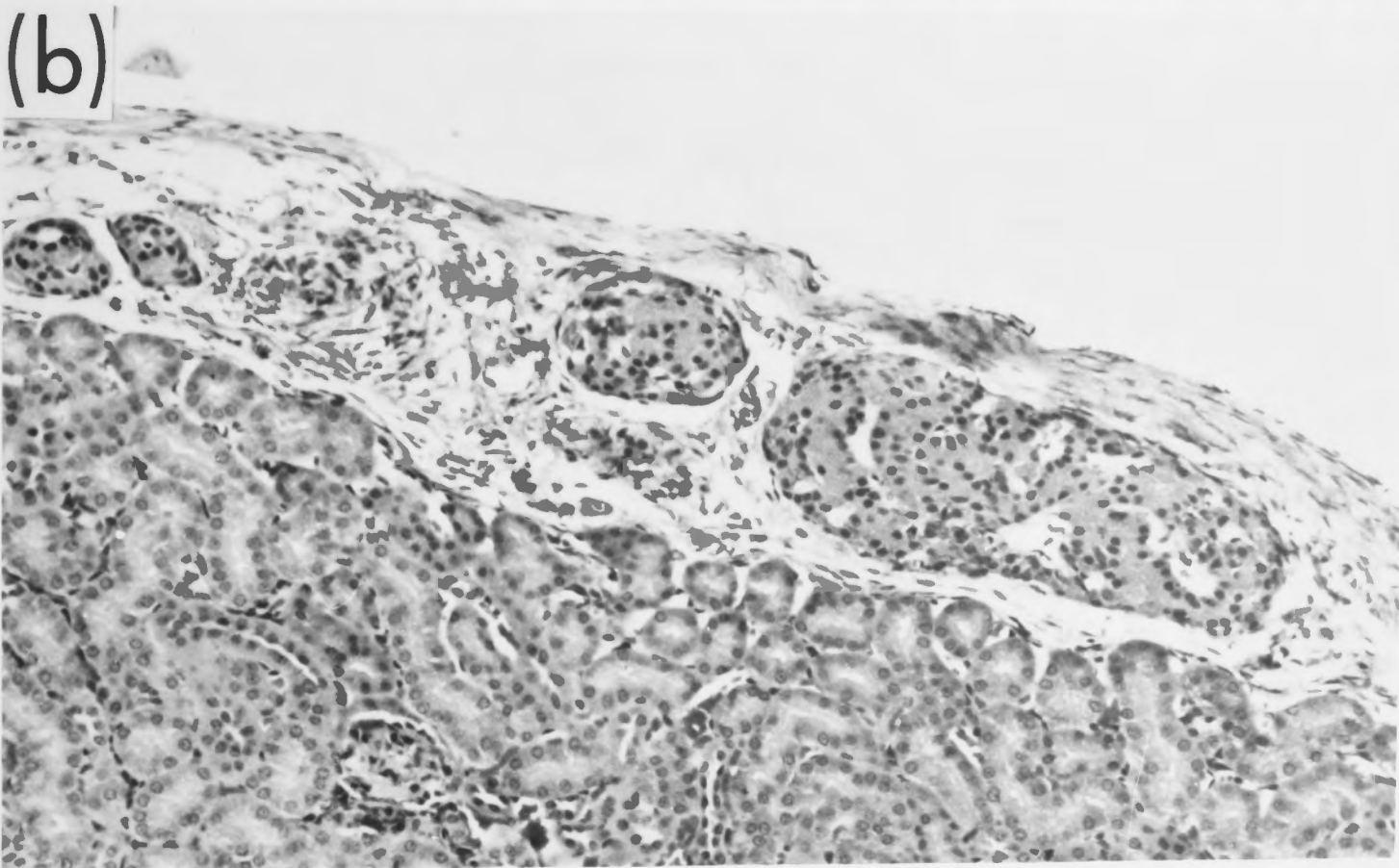
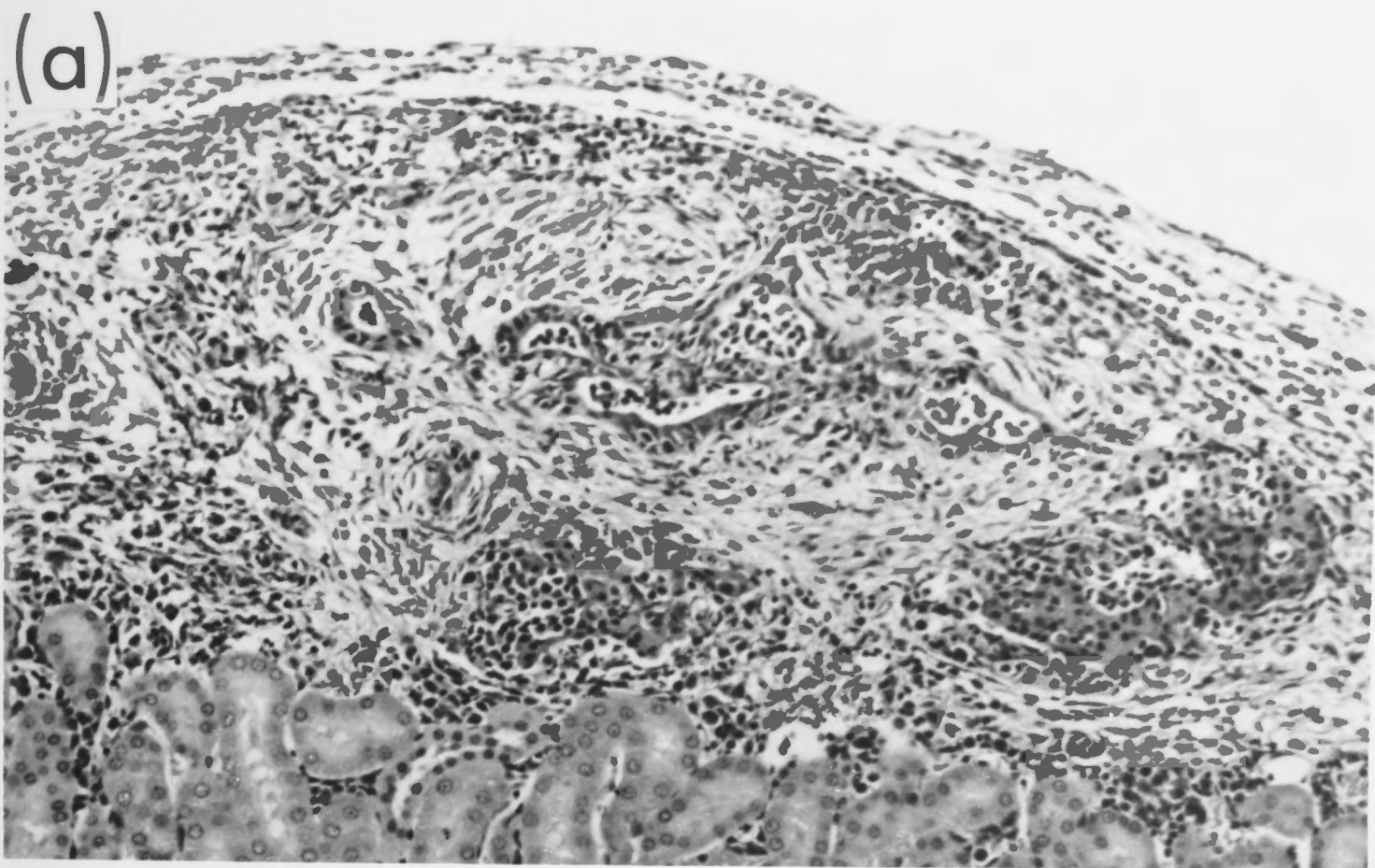


Figure 4.10 (a) Allograft of six 10 day-cultured BALB/c foetal pancreas segments 2 weeks after transplantation under the kidney capsule of a CBA recipient mouse. Note the vigorous allograft response, remnants of damaged islet tissue and some duct epithelium. Haematoxylin and eosin, x 171. (b) Allograft of six 10 day-cultured BALB/c foetal pancreas segments, 4 weeks after transplantation beneath the kidney capsule of an ALS-treated CBA recipient. Note the presence of intact islet tissue and absence of mononuclear cell infiltration. Haematoxylin and eosin, x 168.

histocompatibility barrier. Furthermore, 25% of 20 day-cultured allografts (six segments) showed no evidence of rejection at 12 weeks post-transplantation. The failure of other groups (Hegre *et al.*, 1976a; Garvey *et al.*, 1980b; Mandel and Higginbotham, 1979) to demonstrate a positive effect of organ culture on the histological and/or the functional integrity of foetal pancreas allografts can probably be attributed to the different culture conditions. We have confirmed, in the mouse, the report by Hegre *et al.* (1976a) that organ culture for only 10 days in a high oxygen gas phase was insufficient to prolong the survival of foetal rat pancreas allografts. Our study has illustrated that extension of the culture period to 20 days was required to demonstrate a significant effect of organ culture on the histological appearance of foetal mouse pancreas allografts. Long-term organ culture in a 5% CO₂, 95% air gas phase has been shown to be ineffective in prolonging the survival of foetal pancreas allografts for the time required to reverse Streptozotocin-induced diabetes in both the mouse (Mandel and Higginbotham, 1979) and rat (Garvey *et al.*, 1980b; Morris *et al.*, 1980). Our success in demonstrating reversal of Streptozotocin-induced diabetes following foetal pancreas allotransplantation was obtained by using foetal pancreas tissue cultured for 20 days in a 95% O₂, 5% CO₂ gas phase. It should be noted that the *in vivo* administration of Streptozotocin can result in at least temporary depression of some T cell-mediated immune functions (Mahmoud *et al.*, 1976; Nichols *et al.*, 1979). However, our finding that uncultured foetal pancreas allografts failed to ameliorate the diabetic state in recipient mice suggests that the

successful reversal of Streptozotocin-induced diabetes following allografting of cultured foetal pancreas tissue was attributable to the treatment of the donor tissue in culture, prior to transplantation. Thus, the gas phase and the duration of culture appear to play critical roles in modulating the immunogenicity of foetal pancreas tissue.

Organ culture was much more effective in conditioning isolated adult islets for allotransplantation than in the case of foetal pancreas. Only 7 days of culture in the same 95% O₂, 5% CO₂ gas phase was necessary for successful allotransplantation of adult mouse islets. Thus, we confirmed the preliminary report by Bowen *et al.* (1980) that cyclophosphamide pretreatment of adult donors was not required as an adjunct to organ culture for the reduction of adult islet immunogenicity. In contrast, Andersson and Buschard (1977) were unable to prolong the survival of allogeneic isolated adult islets following organ culture for 10 days in a 5% CO₂, 95% air gas phase and transplantation to diabetic recipient mice. However, Andersson (1979) has reported some evidence indicating that the functional activity of islet allografts was improved by extending the culture period to 28 days. Lacy *et al.* (1979c) failed to show evidence of acceptance of allografts of isolated rat islets following organ culture for up to 28 days in a 5% CO₂, 95% air gas phase. Furthermore, Kedinger and colleagues (1977) were unable to restore normal non-fasting blood glucose levels in rats following allotransplantation of isolated adult islets cultured for 4 days in a 5% CO₂, 95% air gas phase. Taken together, these findings suggest that a high oxygen gas phase also plays an important role in reducing the immunogenicity of isolated

adult islets. This proposal is supported by the demonstration that the modulation of mouse thyroid immunogenicity by organ culture is an oxygen-dependent process (Talmage and Dart, 1978; Talmage, 1980; Section 1.6.3).

We have shown that the mechanism by which the organ culture procedure promoted the acceptance of allogeneic isolated adult islets was not attributable to the loss of tissue antigenicity. The rejection of 7 day-cultured adult islets, following donor-specific stimulation of the host's immune system at the time of transplantation, illustrated that the cultured tissue carried recognizable antigens. This conclusion is supported by the report by Lacy and colleagues (1979c) that 7 day-cultured rat islet allografts, which had survived following transplantation to ALS-treated recipients, were rejected following donor-specific immunization of the hosts. Indeed similar findings were reported for cultured thyroid allografts and xenografts in rodents (Lafferty *et al.*, 1976b; Talmage *et al.*, 1976; Sollinger *et al.*, 1977; Section 1.6.3). The consistent rejection of a proportion of long-term cultured foetal pancreas allografts also suggests that prolonged culture does not modulate the antigenicity of foetal pancreas tissue. Nevertheless, our study has illustrated that the immunogenicity of pancreatic islet tissue can be modified by pretreatment in culture prior to allotransplantation. These findings reinforce the proposal that the effect of organ culture is to remove donor passenger leukocytes from the graft tissue before transplantation (Section 1.6.3).

Our comparative study of the effect of organ culture on the immunogenicity of pancreatic islet tissue indicates that foetal pancreas is highly immunogenic, much more highly immunogenic than isolated adult islets. Using immunosuppression of recipients as an approach to facilitating the acceptance of allografts of pancreatic islet tissue in rats, Garvey *et al.* (1979b) reported that the administration of Cyclosporin A (5 - 40 mg/kg/day) to diabetic rats did not significantly prolong the survival of allogeneic adult islets or foetal pancreas transplanted across the same major histocompatibility barrier. Indeed, both types of allografts were rejected by 13 to 15 days after transplantation. Since similar dosage regimens were effective in prolonging the survival of skin and kidney allografts in the same strain combination, they concluded that both sources of donor pancreatic islet tissue were highly immunogenic. Treatment of transplant recipients with cyclophosphamide, ALS, azathioprine, prednisolone or anti-donor antibody was substantially less effective in prolonging the survival of pancreatic islet tissue allografts than in the case of kidney allografts (Morris *et al.*, 1980). Moreover, the absence of an effect of prolonged organ culture on promoting foetal pancreas allotransplantation was taken as an indication that foetal pancreas tissue may "lack" passenger leukocytes (Morris *et al.*, 1980). Our findings argue against these conclusions and indicate that isolated adult mouse islets are much more weakly immunogenic than foetal mouse pancreas.

We suggest that the greater immunogenicity of foetal pancreas may be attributable to the mass of primitive lymphoid

tissue which contaminates foetal pancreas explants (Section 3.2.1). This lymphoid component essentially represents an appendage of passenger leukocytes supplementing the population of blood cells carried within the tissue vasculature. The selective removal of this contaminating tissue by dissection was hindered by the fact that discrete lymphoid tissue could not be identified with the aid of a stereomicroscope. Unlike uncultured isografts (Section 3.2.2), isografts of cultured foetal mouse pancreas did not develop discrete lymph node structures. This finding indicates that organ culture damaged the associated lymphoid compartment, thus supporting the premise that organ culture in a high oxygen gas phase can damage passenger leukocytes (Lafferty *et al.*, 1976a, 1976b; Section 1.6.3). Even so, it is possible that residual lymphoid cells persisted and were sufficient to trigger allograft rejection. Indeed, Lane and co-workers (1975) demonstrated that dissociated rat fetuses and dissociated foetal skin cells at 19 days but not 15 days of gestation, expressed the capacity to stimulate allogeneic adult lymphocytes to proliferate in mixed cell cultures. These findings suggested that stimulator cell function developed late in gestation. The immunogenic potency of foetal mouse pancreas at 17 days of gestation is consistent with this conclusion.

Finke and colleagues (1979) have reported that preparations of adult rat islets contain small lymph nodes which represent a rich source of passenger leukocytes. However, careful selection of the islets by hand-picking, to avoid this source of lymphoid contamination, facilitated the allotransplantation of rat islets prepared from donors pretreated

with irradiation and silica (Lacy *et al.*, 1979b). We have shown that organ culture alone for 7 days in a 95% O₂, 5% CO₂ gas phase is sufficient pretreatment to prevent the rejection of hand-picked adult mouse islets transplanted across a major histocompatibility difference. This finding indicates that such isolated adult islets are weakly immunogenic. On the basis of these observations, it is likely that purification of islet preparations by hand-picking, and hence the removal of contaminating lymph nodes, represents a major step towards obtaining allotransplantable islets.

Our findings illustrate that tissue-specific differences in immunogenicity exist and suggest that the phenomenon is related to the degree of contamination by lymphoreticular elements. Consequently, there is no general rule that defines the culture conditions required to facilitate allograft acceptance. Each tissue must be examined individually to determine the optimal culture conditions required to obtain successful allotransplantation. Our study has indicated that organ culture in a high oxygen gas phase is not as efficient in modulating the immunogenicity of foetal pancreas tissue as it is in the case of isolated adult islets.

In the case of mouse thyroid, the effect of organ culture on allograft survival was potentiated by pretreatment of donor animals with 300 mg/kg cyclophosphamide on days -4 and -2 prior to explantation of the donor tissue (Lafferty *et al.*, 1976a; Section 1.6.3). This effect of cyclophosphamide was attributed to its leukotoxic activity and hence to the capacity to destroy passenger leukocytes. However, donor pretreatment alone with cyclophosphamide resulted in only a modest increase

in thyroid allograft survival (Lafferty *et al.*, 1976a; Section 1.6.3). The approach of donor pretreatment with agents that induce leukopenia is not suitable in the case of foetal donors; there is the practical problem of drug administration and the likelihood of drug toxicity. Nevertheless, Jonasson (1979) reported that pretreatment of monkey foetuses *in utero* with cyclophosphamide for 5 days followed by organ culture of foetal pancreas explants for 7 to 9 days did not prolong the survival of foetal pancreas allografts transplanted either to siblings or to the maternal host. Garvey *et al.* (1980a) also demonstrated that cyclophosphamide pretreatment of pregnant rats 24 hours prior to removal of the foetuses had little effect in prolonging foetal pancreas allograft survival.

Treatment of transplant recipients with antilymphocyte xenosera (ALS) has been investigated as an alternative option for prolonging foetal pancreas allograft survival. Morris and colleagues (1980) reported that short-term treatment of transplant recipients with ALS significantly prolonged the survival of foetal rat pancreas allografts in selected F_1 \rightarrow parent (P_1 or P_2) strain combinations. Using a similar schedule for ALS treatment, we were unable to demonstrate long-term islet survival in foetal mouse pancreas allografts. However, our data suggest that there was a synergistic effect of organ culture and ALS treatment on prolonging islet survival in 10 day-cultured allografts. Lacy *et al.* (1979a), working with isolated adult rat islets, demonstrated that organ culture in combination with ALS-treatment of transplant recipients indefinitely prolonged islet allograft survival.

Furthermore, they suggested that the ALS treatment may act not only to immunosuppress the transplant recipients temporarily but also to remove residual passenger leukocytes from the transplant tissue (Lacy *et al.*, 1979c). Conversely, in our study, islet survival in 10 day-cultured foetal pancreas allografts was only temporarily prolonged; graft rejection was evident by 8 weeks post-transplantation. This finding suggested that the stimulatory capacity of 10 day-cultured foetal pancreas tissue persisted beyond the time of host recovery from ALS-induced immunosuppression. In relation to this point, our data indicate that ALS was not effective in further reducing the immunogenicity of foetal mouse pancreas cultured for up to 20 days in a 95% O₂, 5% CO₂ gas phase.

The evidence presented in this chapter indicates that foetal mouse pancreas is highly immunogenic. We have proposed that this property is probably attributable to the mass of primitive lymphoid tissue which contaminates foetal pancreas explants. This associated lymphoid component offers some explanation for the difficulties in preventing allograft rejection either by means of organ culture or recipient immunosuppression.

4.4 SUMMARY

Organ culture for 20 days in a 95% O₂, 5% CO₂ gas phase significantly improved the histological integrity of foetal pancreas allografts. At 12 weeks post-transplantation, 25% of allografts consisting of six 20 day-cultured foetal pancreas segments showed no evidence of rejection. The organ culture procedure was much more effective in modulating the immunogenicity of isolated adult islets than in the case of the

foetal pancreas; only 7 days of culture in the same gas phase was necessary to obtain indefinite survival of allogeneic isolated adult mouse islets following transplantation across the same major histocompatibility barrier. We have proposed that the much higher immunogenicity of foetal pancreas tissue is probably related to the contamination of explants by primitive lymphoid tissue.

Allografts consisting of six 20 day-cultured segments (tissue from two foetal pancreas donors), and transplanted beneath the kidney capsule, successfully reversed Streptozotocin-induced diabetes in a proportion of recipient mice.

The survival of uncultured and cultured foetal pancreas allografts was not improved in the long-term by treatment of transplant recipients with ALS.

In the following chapter (Chapter 5), we extend our investigation of the immunogenic component of foetal pancreas tissue.

3.1 INTRODUCTION

In the previous chapter, we presented evidence indicating that foetal pancreas is much more highly immunogenic than adult pancreas. Whereas adult islets required only 7 days of organ culture in 35% O_2 , 5% CO_2 for successful allograftation, 17 to 20 days of culture in the same gas phase were necessary to show evidence of acceptance of a proportion of foetal pancreas allografts.

The concept of immunogenicity is based on the premise that passages of cells present in transplant tissue are responsible for stimulating allograft rejection (Section 1.6.1).

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(Section 1.5.1). The purpose of the organ culture technique in isolating tissue immunogenicity has been explained in these terms (Section 1.6.1). Preparation of islets isolated from the adult pancreas by collagenase digestion are contaminated with very small lymph nodes which represent a rich source of immunogenic cells (Section 1.5.1). However, we have demonstrated that when adult islets which have been subjected to a similar procedure are used, they are immunogenic (Section 1.5.1).

The histological evidence which we presented in Chapter 1 (Section 1.2.1, Section 1.2.2) indicated that foetal mouse pancreas explants contain substantial quantities of primitive lymphoid tissue. This lymphoid tissue appears to be derived from the mesentery which surrounds the organ in vivo (Section 1.3.1). On the basis of our earlier conclusions,

5.1 INTRODUCTION

In the previous chapter, we presented evidence indicating that foetal pancreas is much more highly immunogenic than islets isolated from the adult pancreas. Whereas adult islets required only 7 days of organ culture in 95% O₂, 5% CO₂ for successful allotransplantation, 17 to 20 days of culture in the same gas phase were necessary to show evidence of acceptance of a proportion of foetal pancreas allografts.

Our concept of tissue immunogenicity is based on the premise that passenger leukocytes present in transplant tissue are responsible for stimulating allograft rejection (Section 1.6.1; Section 1.6.3). A corollary of this postulate is that reduction of tissue immunogenicity should accompany removal of the passenger leukocyte population prior to transplantation (Section 1.6.1). The success of the organ culture technique in modulating tissue immunogenicity has been explained in these terms (Section 1.6.3). Preparations of islets isolated from the adult pancreas by collagenase digestion are contaminated with very small lymph nodes which represent a rich source of leukocytes (Finke *et al.*, 1979). However, we have demonstrated that clean adult mouse islets which have been handpicked to avoid lymphoid contaminants are weakly immunogenic (Section 4.2.3).

The histological evidence which we presented in Chapter 3 (Section 3.2.1; Section 3.2.2) indicated that foetal mouse pancreas explants contain substantial quantities of primitive lymphoid tissue. This lymphoid tissue appears to be derived from the mesentery which surrounds the organ *in situ* (Section 3.3). On the basis of our earlier postulate, we

have proposed that this associated lymphoid component is responsible for the high immunogenicity of foetal pancreas tissue and for the limited success of the organ culture technique to facilitate allotransplantation (Section 4.3). An alternative explanation for the marked difference in the immunogenicity of foetal pancreas and isolated adult islets is that there exists an intrinsic difference between foetal and adult islets.

The isolation of foetal islets has been demonstrated using the perinatal rat pancreas (Heinze and Steinke, 1971; Hellerström *et al.*, 1979; Hegre *et al.*, 1981) and foetal human pancreas (Goldman and Colle, 1976) as donor tissue. However, information on the immunogenicity of isolated foetal islets has not been reported. We therefore set out to investigate whether foetal islets could be isolated from mouse foetal pancreas and associated primitive lymphoid components.

In this chapter we describe the isolation and properties of a new entity, the foetal 'proislet'. We investigate the capacity of 'proislets' to differentiate into islets and we document their histological and functional properties following isotransplantation. Finally we examine whether isolated foetal mouse proislets are more or less immunogenic than uncultured and 10 day-cultured foetal pancreas tissue.

5.2 RESULTS

5.2.1 Digestion of mouse foetal pancreas

Our first aim was to determine whether foetal islets could be isolated from mouse foetal pancreas. Foetal pancreases were subjected to a brief collagenase digestion (Section 2.4) and the digested tissue was cultured in a 5% CO₂,

95% air gas phase. A 95% O₂, 5% CO₂ atmosphere was not used in this instance because experience with adult islets has indicated that a high oxygen gas phase is toxic for single isolated islets (Lacy *et al.*, 1979a; Bowen *et al.*, 1980).

At various time intervals, cultured tissue samples were taken for histological analysis. Histological examination of freshly digested (0 day-cultured) BALB/c foetal pancreas demonstrated that the digest consisted predominantly of differentiated acinar tissue, considerably disrupted after collagenase treatment, and a small amount of undifferentiated tissue (Fig. 5.1). After 2 days in culture, only remnants of acinar tissue remained and by 4 days essentially all acinar tissue had completely disappeared. The 4 day-cultured digest contained some single cells but consisted mainly of discrete ovoid tissue pieces containing undifferentiated cells, some duct-like epithelial components, connective tissue, and occasionally peripheral regions of cellular organization which resembled endocrine tissue (Fig. 5.2). Evidence for cellular proliferation was provided by the presence of mitotic figures in some sections. On the basis of the evidence to be presented in this chapter, we have called these units of undifferentiated tissue 'proislets'. Although no further *in vitro* maturation of 'proislets' into histologically identifiable islets was observed after prolonged culture (up to 21 days), peripheral regions of some 'proislets' showed evidence of further differentiation. However, at no stage did isolated 'proislets' stain to any significant extent with aldehyde fuchsin.

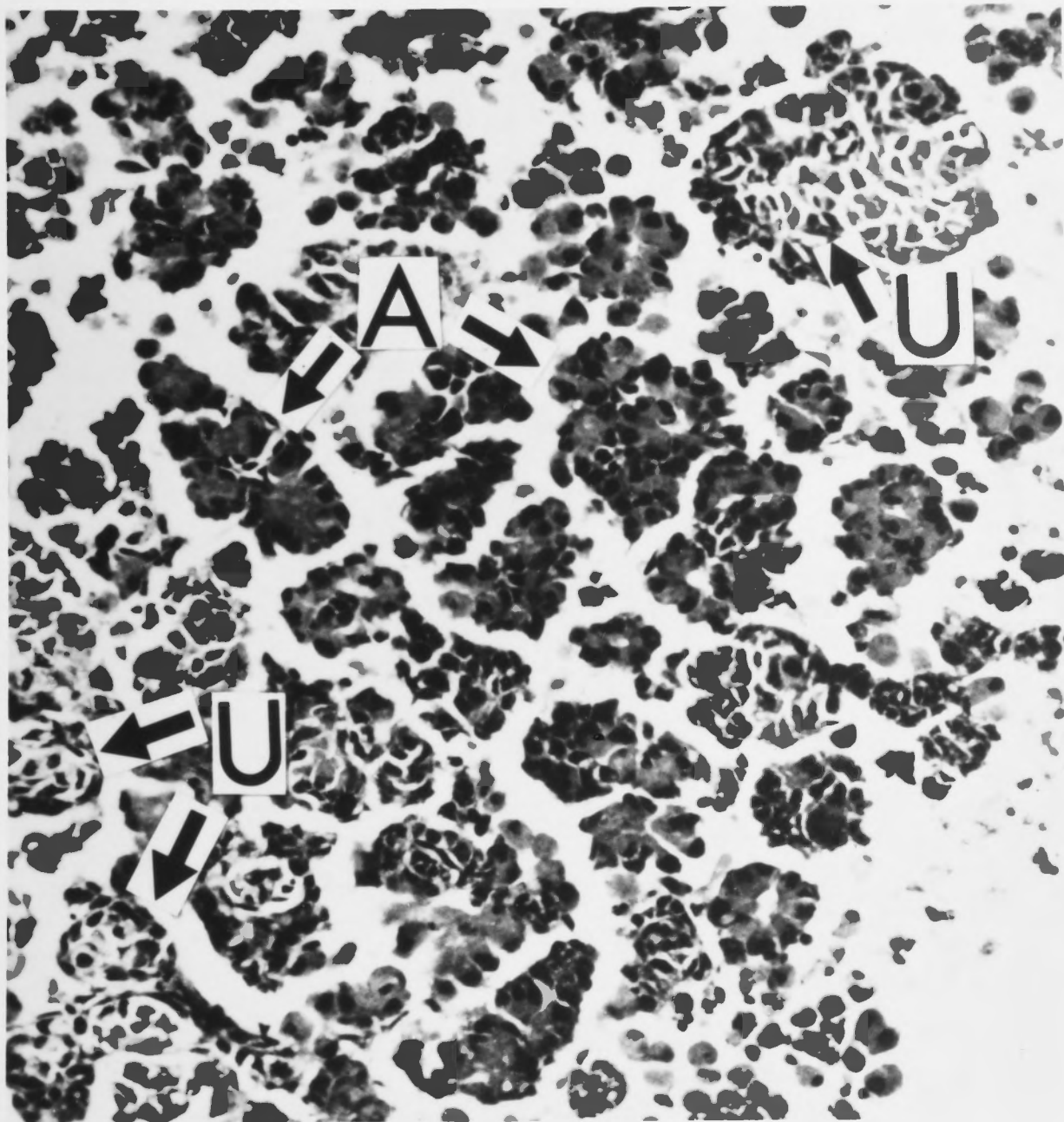


Figure 5.1 Uncultured BALB/c foetal pancreas digest. Note
disrupted acinar tissue (A) and small amount of
undifferentiated tissue (U). Haematoxylin and eosin,
x 383.

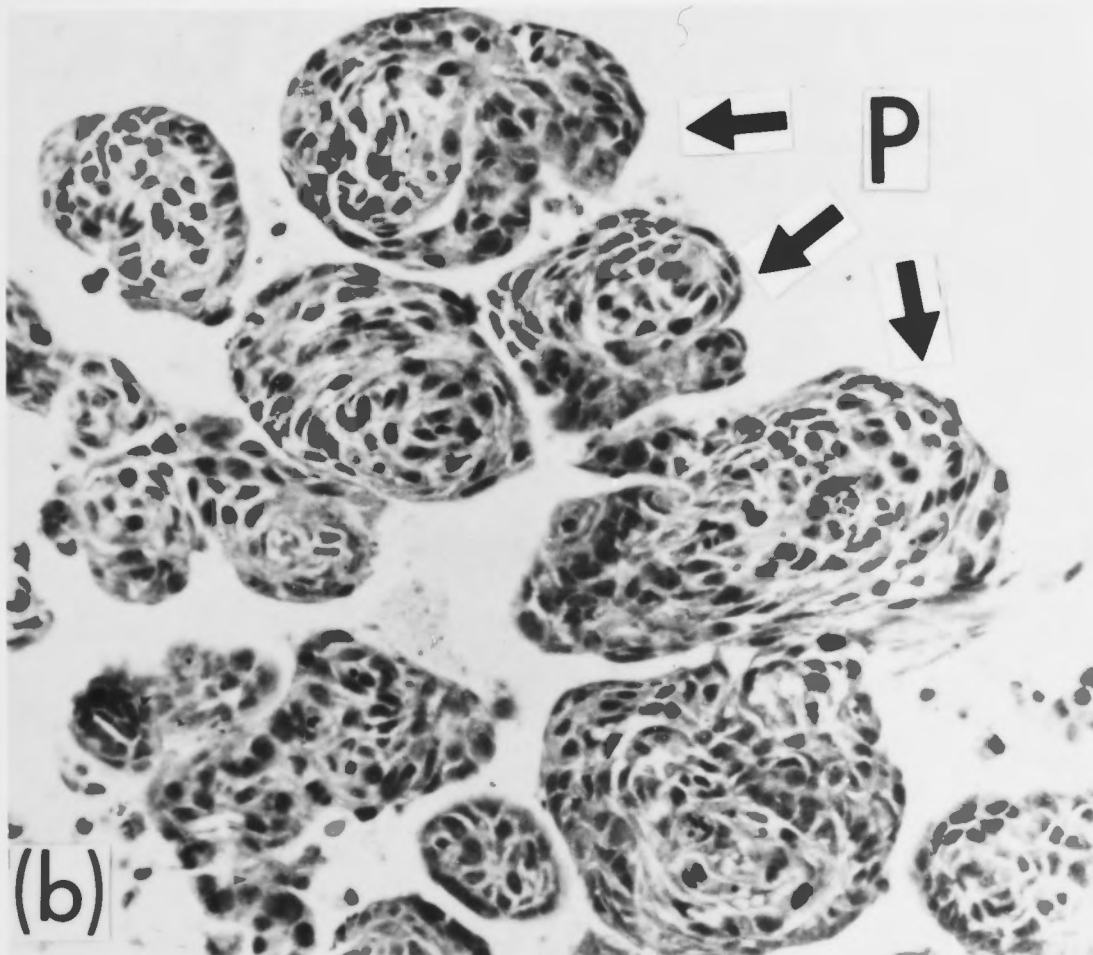


Figure 5.2 Proislets isolated from 4 day-cultured BALB/c foetal pancreas digest. Panel (a) shows proislet (P) containing columnar epithelium (E), undifferentiated tissue (U) and small region of differentiating tissue (D). Haematoxylin and eosin, x 335. Panel (b) shows several proislets (P) composed of undifferentiated tissue. Haematoxylin and eosin, x 335.

5.2.2 Isotransplantation of foetal mouse 'proislets'

To examine the capacity of foetal 'proislets' to develop into fully differentiated islets, isolated BALB/c 'proislets' were transplanted under the kidney capsule of normal isogeneic recipient mice. At 2, 4 and 12 weeks post-transplantation, groups of mice were sacrificed and the isografts were taken for histological analysis.

Table 5.1 shows that seven of seven isografts taken at 2, 4 and 12 weeks after transplantation contained well vascularized, differentiated islets (Fig. 5.3). The islet tissue stained positively with aldehyde fuchsin, indicating that granulated β cells were present. In addition, some duct epithelium, a small amount of undifferentiated tissue and occasionally a few mononuclear cells were identified.

Thus, 'proislets' are so called because following isotransplantation they differentiate into histologically-recognizable islets. It is apparent that *in vitro* proislets represent precursor islet tissue.

5.2.3 Function of proislet isografts

In view of the islet development which occurred following proislet transplantation, we investigated the capacity of proislet isografts to reverse Streptozotocin-induced diabetes. Proislets harvested from 8 foetal BALB/c pancreases were transplanted in 4 plasma clots (Section 2.10) under the kidney capsule of diabetic, isogeneic mice. The group contained a total of 5 transplant recipients and the mice were maintained daily with 0.8 units of Ultralente insulin for 22-24 days after transplantation (see Section 2.17).

TABLE 5.1

Histological assessment of foetal proislet isografts^a

Time post-transplant (weeks)	Total No. grafts	No. grafts showing differentiated islets	% grafts showing islet development
2	7	7	100
4	7	7	100
12	7	7	100

a. Isografts consisted of proislets harvested from two BALB/c foetal pancreases (Section 2.10).

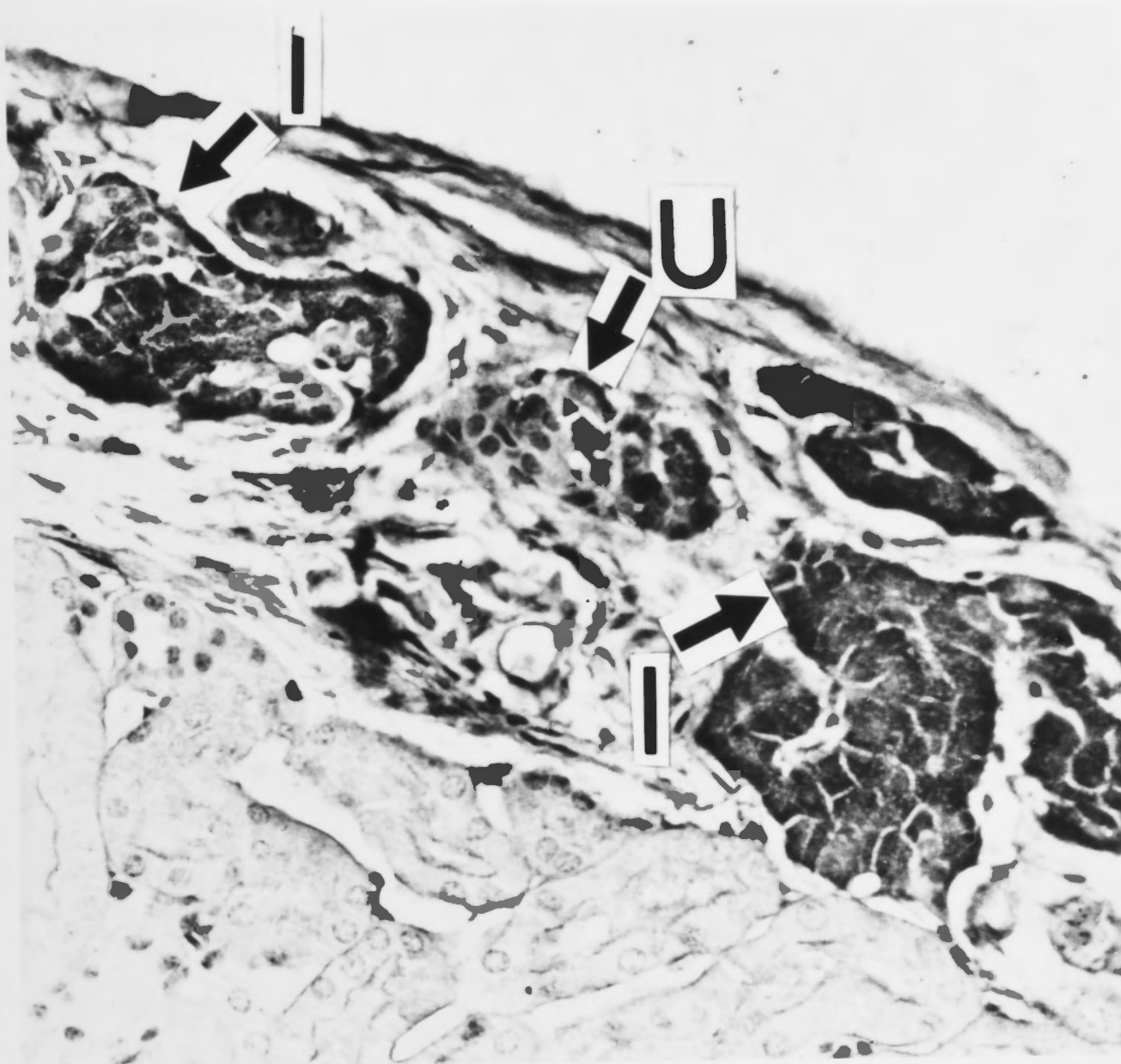


Figure 5.3 Isograft of BALB/c proislets 4 weeks after transplantation under the kidney capsule of a BALB/c recipient mouse. Note the presence of well differentiated, aldehyde fuchsin-positive islets (I) and a small amount of undifferentiated tissue (U). Aldehyde fuchsin, x 408.

Figure 5.4(a) shows that foetal mouse proislets can reverse Streptozotocin-induced diabetes and return blood glucose levels to within the normal range by as early as 36-39 days after transplantation. There was considerable variation in the functional response of proislet isografts; in some mice, normoglycaemia was not reached until 197-286 days after transplantation. Graft function was accompanied by a steady increase in body weight. In addition, aglycosuria was restored in two of the four mice in which normoglycaemia was re-established (Table 5.2). The urine glucose output of the other two animals remained just outside the normal range. Nevertheless, these levels were approximately 3 orders of magnitude lower than for control diabetic mice. Surgical removal of the graft-bearing kidney confirmed that maintenance of normoglycaemia had been graft-dependent. The return to a diabetic state was accompanied by a rapid loss in body weight. Histological examination of these grafts showed the presence of massive, well differentiated islets (Fig. 5.5). Figure 5.4(b) shows that in one mouse, the blood glucose levels fluctuated within the hyperglycaemic range for 380 days, at which point the study was terminated. The marked increase in body weight which occurred early after transplantation suggests that this proislet isograft conveyed at least some beneficial effect. Macroscopic examination of the graft at 380 days post-transplantation revealed a small number of well-vascularized islets. Thus, it appeared that the number of proislets grafted had been insufficient to achieve complete control of blood glucose levels within the observation period.

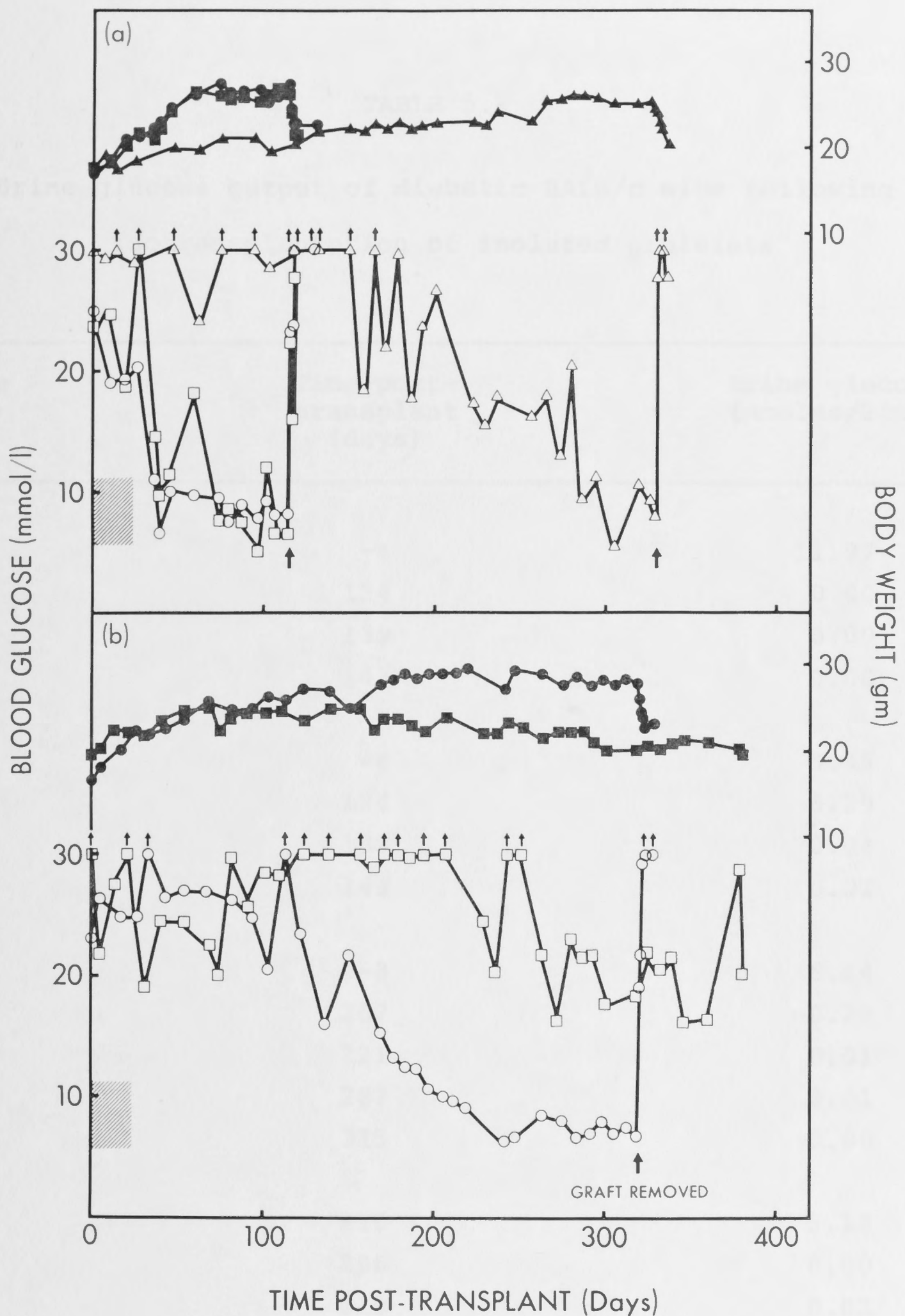


Figure 5.4 Non-fasting blood glucose levels (open symbols) and body weight (closed symbols) of diabetic BALB/c mice following isotransplantation of proislets isolated from 8 foetal pancreases, under the kidney capsule. The shaded region represents the 95% probability interval for the non-fasting blood glucose levels of normal BALB/c mice. Grafts were removed by excision of the graft-bearing kidney. Panels (a) and (b) show the data for three and two transplant recipients respectively.

TABLE 5.2

Urine glucose output of diabetic BALB/c mice following
isotransplantation of isolated proislets^a

Mouse Code	Time post- transplant (days)	Urine glucose ^b (mmoles/24 hr)
(1)	-4	11.97
	124	0.00
	139	0.00
	143	0.00
(2)	-4	9.45
	124	0.29
	139	0.04
	143	0.01
(3)	-3	6.64
	207	0.22
	221	0.01
	287	0.01
	315	0.00
(4)	-16	5.18
	296	0.00
	318	0.02
	324	0.03

a. The table contains data for the 4 transplant recipients which became normoglycaemic.

b. Urine glucose output for normal BALB/c mice was 0.00 mmoles glucose/24 hours; urine glucose levels for control diabetic BALB/c mice were ≥ 9.38 mmoles glucose/24 hours (Section 2.18).

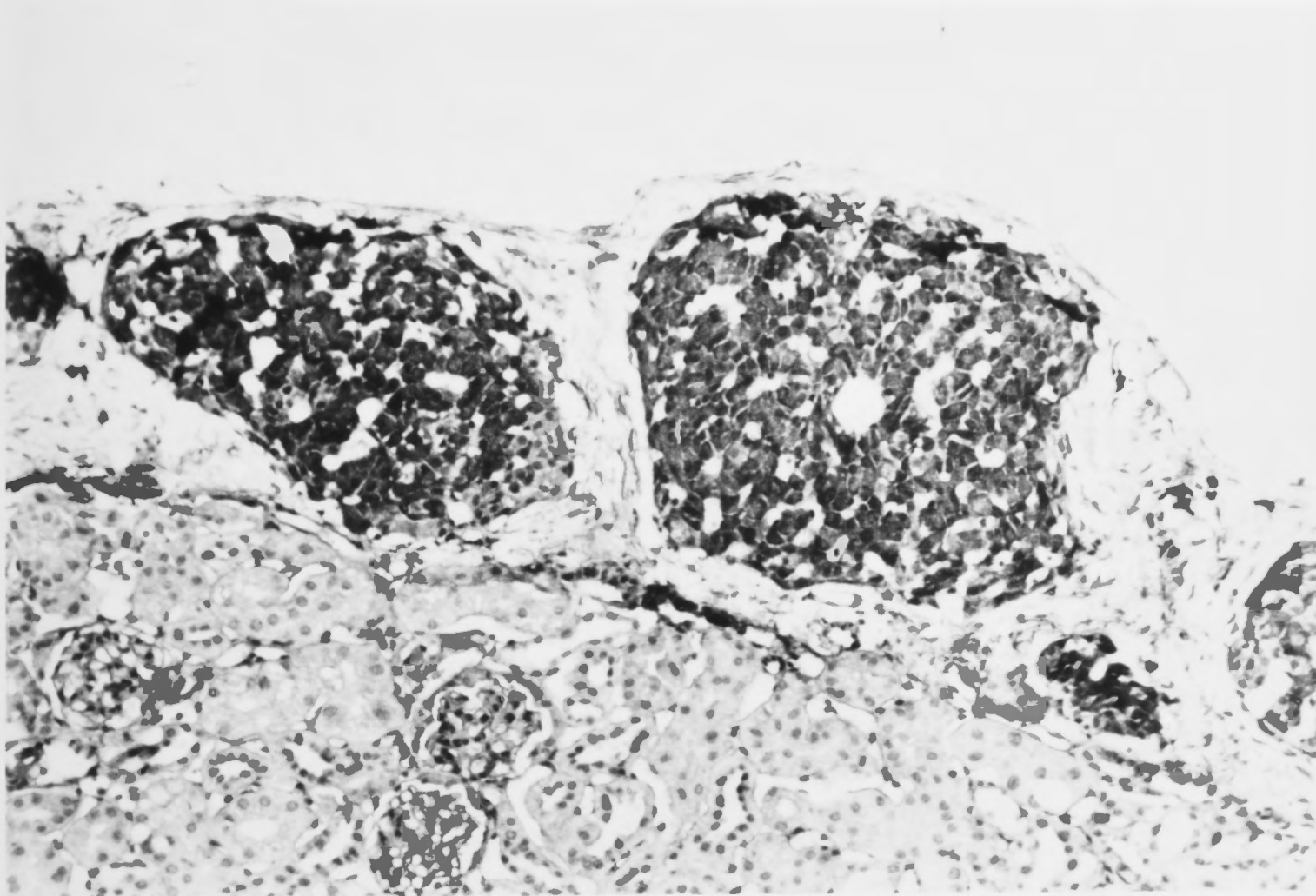


Figure 5.5 Isograft of BALB/c proislets harvested from eight foetal pancreases, at 334 days after transplantation beneath the kidney capsule of a diabetic BALB/c recipient mouse. Note massive, well differentiated islets with β cell granulation. Aldehyde fuchsin, x 161.

This study demonstrated that isografts of foetal mouse proislets can reverse Streptozotocin-induced diabetes. Furthermore, these findings are consistent with the histological data which indicate that, following transplantation, proislets develop into differentiated islets with granulated β cells.

5.2.4 Allotransplantation of isolated proislets

To assess the immunogenicity of foetal mouse proislets, BALB/c proislets were transplanted under the kidney capsule of normal allogeneic CBA recipients. The integrity of proislet allografts was examined histologically at 2, 4 and 12 weeks post-transplantation. The criteria for allograft survival were the absence of infiltrating mononuclear cells at the graft site and the presence of intact islets. The survival of proislet allografts was compared to that of uncultured and 10 day-cultured foetal pancreas allografts transplanted across the same major histocompatibility barrier.

Table 5.3 shows that six of nine, and five of nine proislet allografts at 2 and 4 weeks after transplantation respectively showed no evidence of rejection. Histologically, surviving allografts showed no mononuclear cell infiltration and contained intact, differentiated islet tissue with granulated β cells (Fig. 5.6(a)). Occasionally duct epithelium and a small amount of undifferentiated tissue was also observed. The remaining allografts showed obvious signs of rejection; histological examination revealed that two of nine grafts at 2 weeks post-transplantation contained a mononuclear cell infiltrate and some damaged islet tissue (Fig. 5.6(b)). One of nine and three of nine proislet allografts at

TABLE 5.3

Survival of proislet and foetal pancreas allografts

Allograft ^a	Time post-transplant (weeks)	Total No. grafts examined	No. grafts with histological score ^b					% grafts surviving ^c
			++++	+++	++	+	0	
Isolated proislets	2	9	6 ^{d,f,g}	-	2	1	-	67
	4	9	5 ^e	-	-	3	1	56
	12	10	1 ^{d,e}	1		4	4	10
10 day-cultured foetal pancreas	2	16	- ^g	-	5	11	-	0
Uncultured foetal pancreas	2	9	- ^f	-	-	9	-	0

a. Allografts consisted of proislets harvested from two foetal pancreases, six foetal pancreas segments cultured for 10 days in 95% O₂, 5% CO₂ or one uncultured foetal pancreas.

b. Histological score key:

++++ Intact islets without mononuclear cell infiltration at the graft site.

+++ Intact islets with mononuclear cell infiltration at the graft site but no infiltration into islets.

++ Mononuclear cell infiltration and damaged islet tissue present.

+ Mononuclear cell infiltration and scar tissue.

0 Fibrotic scar tissue without mononuclear cell infiltration.

c. Criteria for allograft survival were the presence of intact islets and the absence of mononuclear cell infiltration at the graft site.

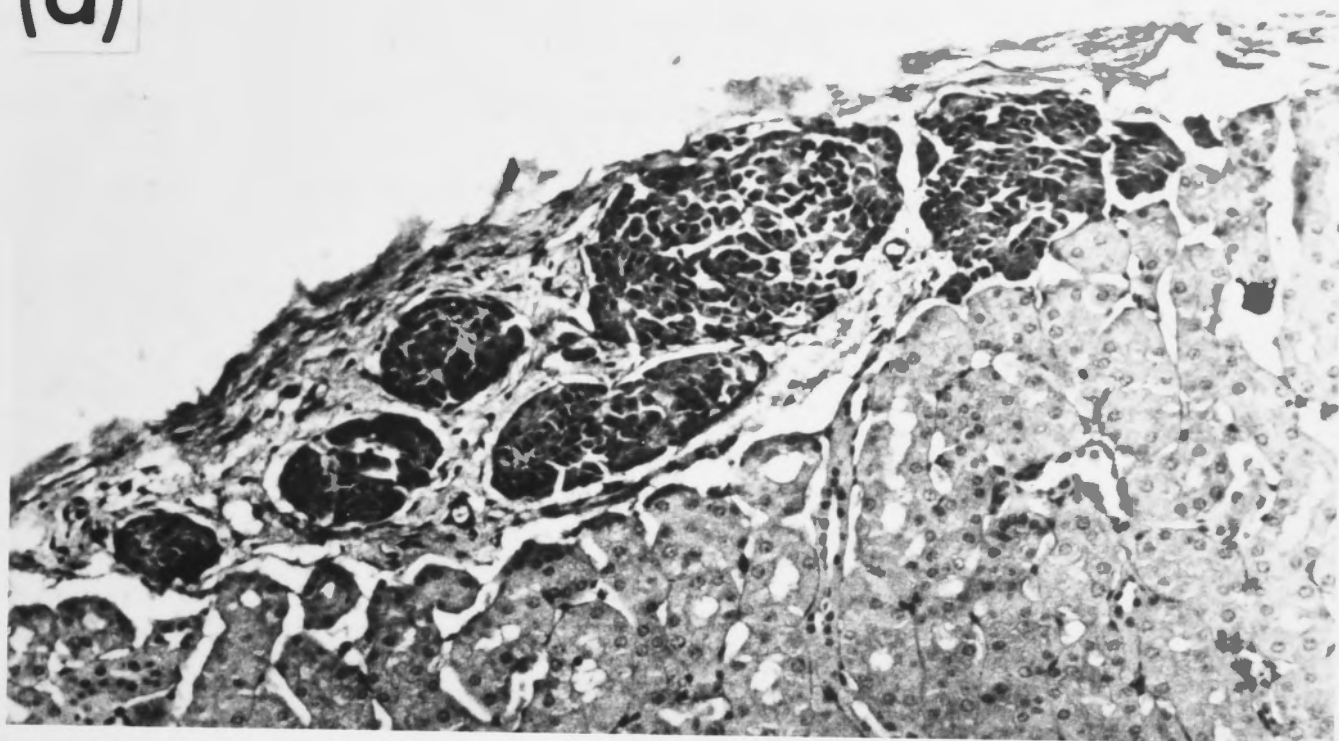
d. P = 0.0348 (Exact probability test; Swinscow, 1980b).

e. P = 0.0991 (Exact probability test; Swinscow, 1980b).

f. P = 0.0090 (Exact probability test; Swinscow, 1980b).

g. P = 0.0009 (Exact probability test; Swinscow, 1980b).

(a)



(b)

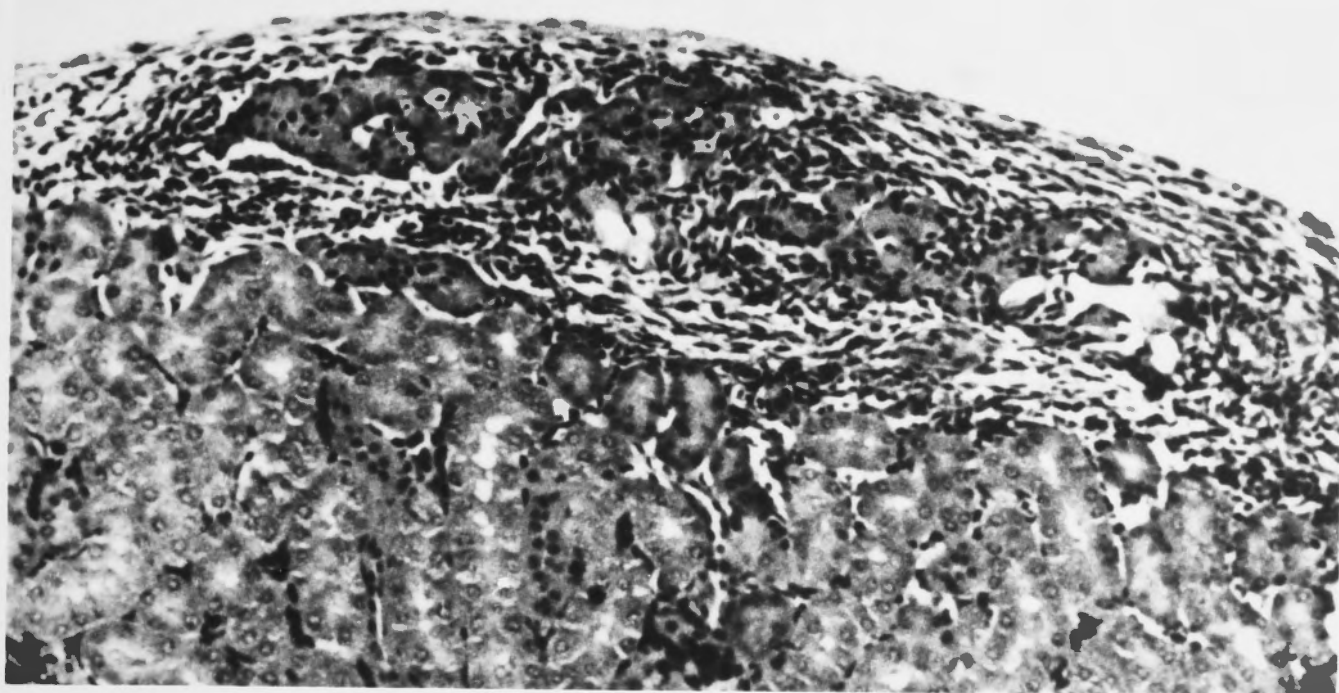


Figure 5.6 (a) Allograft of BALB/c ($H-2^d$) proislets 4 weeks after transplantation under the kidney capsule of a CBA ($H-2^k$) recipient. Note the development of intact differentiated islets with granulated β cells, and absence of any rejection response. Aldehyde fuchsin, x 162. (b) Allograft of BALB/c proislets 2 weeks after transplantation beneath the kidney capsule of a CBA recipient mouse. Note mononuclear cell infiltration and remnants of damaged islet tissue. Haematoxylin and eosin, x 158.

2 and 4 weeks post-transplantation, respectively, showed mononuclear cell infiltration, scar tissue and no evidence of islet tissue. The remaining allograft at 4 weeks post-transplantation contained only a few mononuclear cells and consisted mainly of fibrotic scar tissue. At 12 weeks post-transplantation, one of ten proislet allografts contained intact, well developed islet tissue and showed no mononuclear cell infiltration (Fig. 5.7). Another allograft contained intact islets in the presence of a mild mononuclear cell infiltrate. However, there was no evidence of cellular infiltration into the islets (Fig. 5.8(b)). Macroscopically, this graft was well vascularized and islet development was evident (Fig. 5.8(a)). Four of the ten proislet grafts were completely rejected; no islet tissue was identified and only scar tissue and a light mononuclear cell infiltrate remained at the site of transplantation. The remaining grafts predominantly consisted of scar tissue and a few mononuclear cells. It is unlikely that technical failure contributed to this latter result since all proislet isografts contained differentiated islet tissue (Section 5.2.2). On this basis, we suggest that such allografts were probably rejected early in the post-transplant period. Statistical analyses showed that the proportion of proislet allografts surviving at 12 weeks was significantly lower than the survival of allografts at 2 weeks post-transplantation ($P = 0.0348$). However, there was no significant difference at the 5% level between the survival of proislet allografts at 4 and 12 weeks after transplantation ($P = 0.0991$). It is possible, therefore, that some proislet allografts undergo chronic rejection.

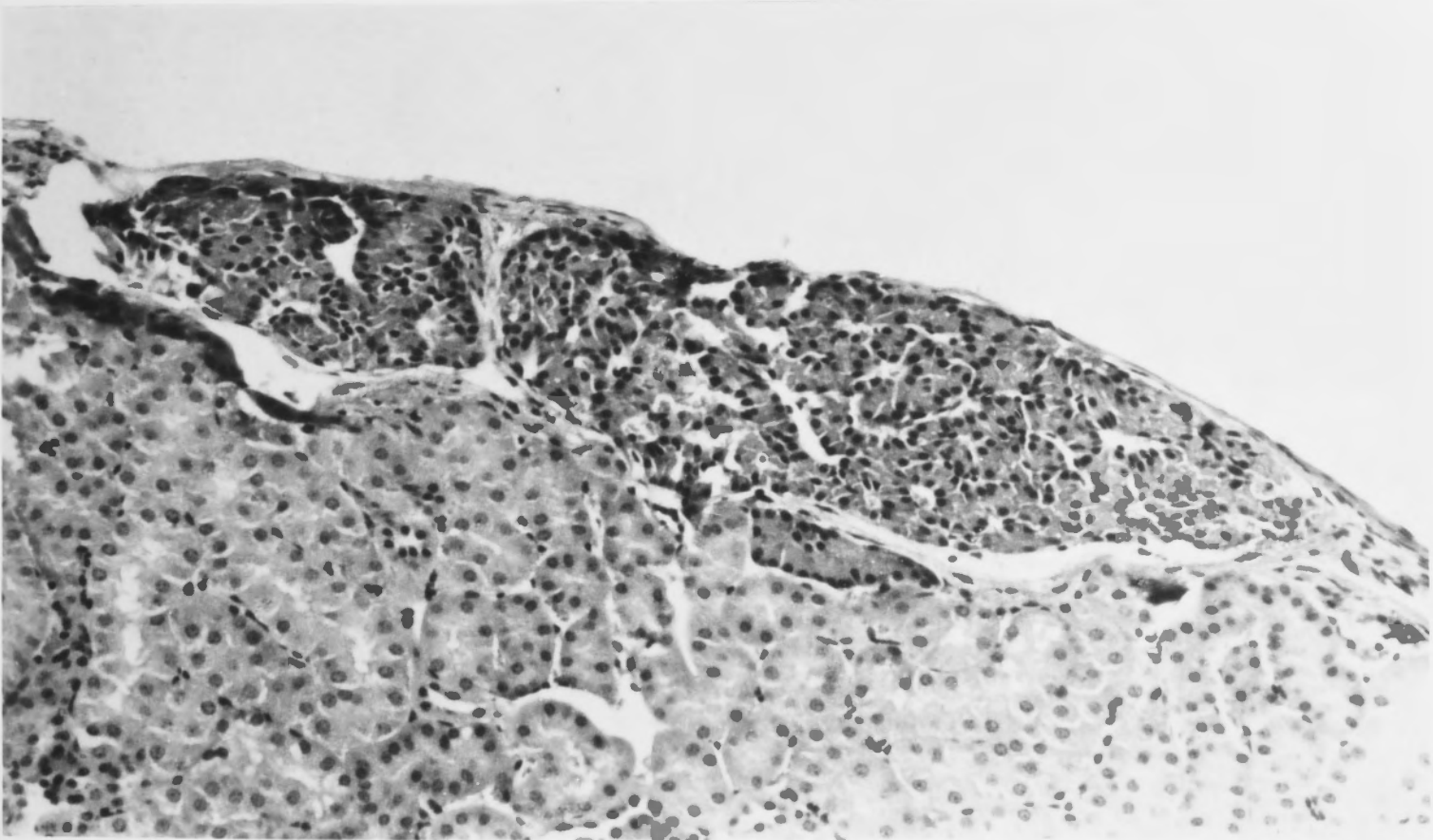


Figure 5.7 Allograft of BALB/c proislets 12 weeks post-transplantation under the kidney capsule of a CBA recipient mouse. Note the presence of intact, well differentiated islet tissue and lack of mononuclear cell infiltration. Haematoxylin and eosin, x 163.

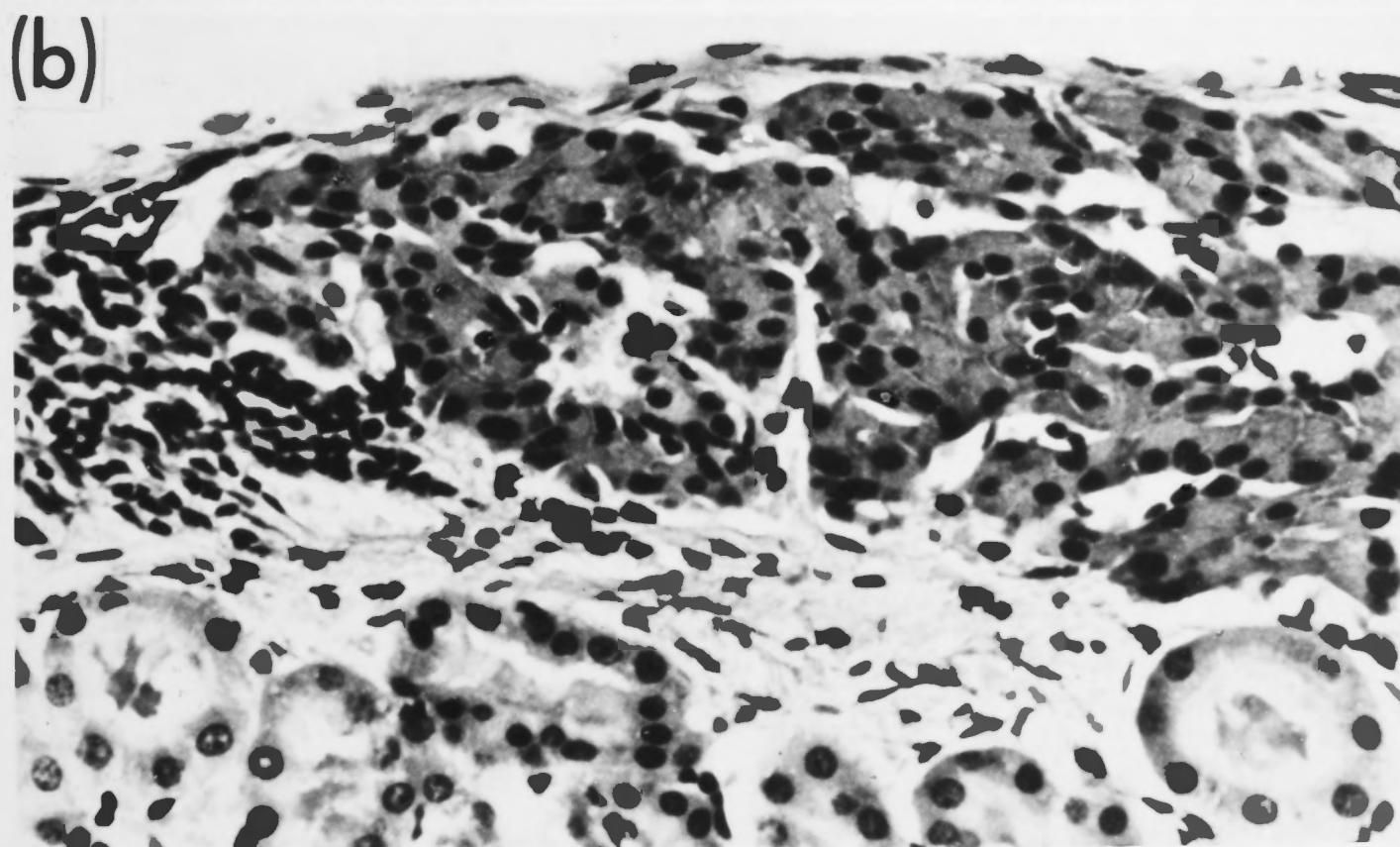
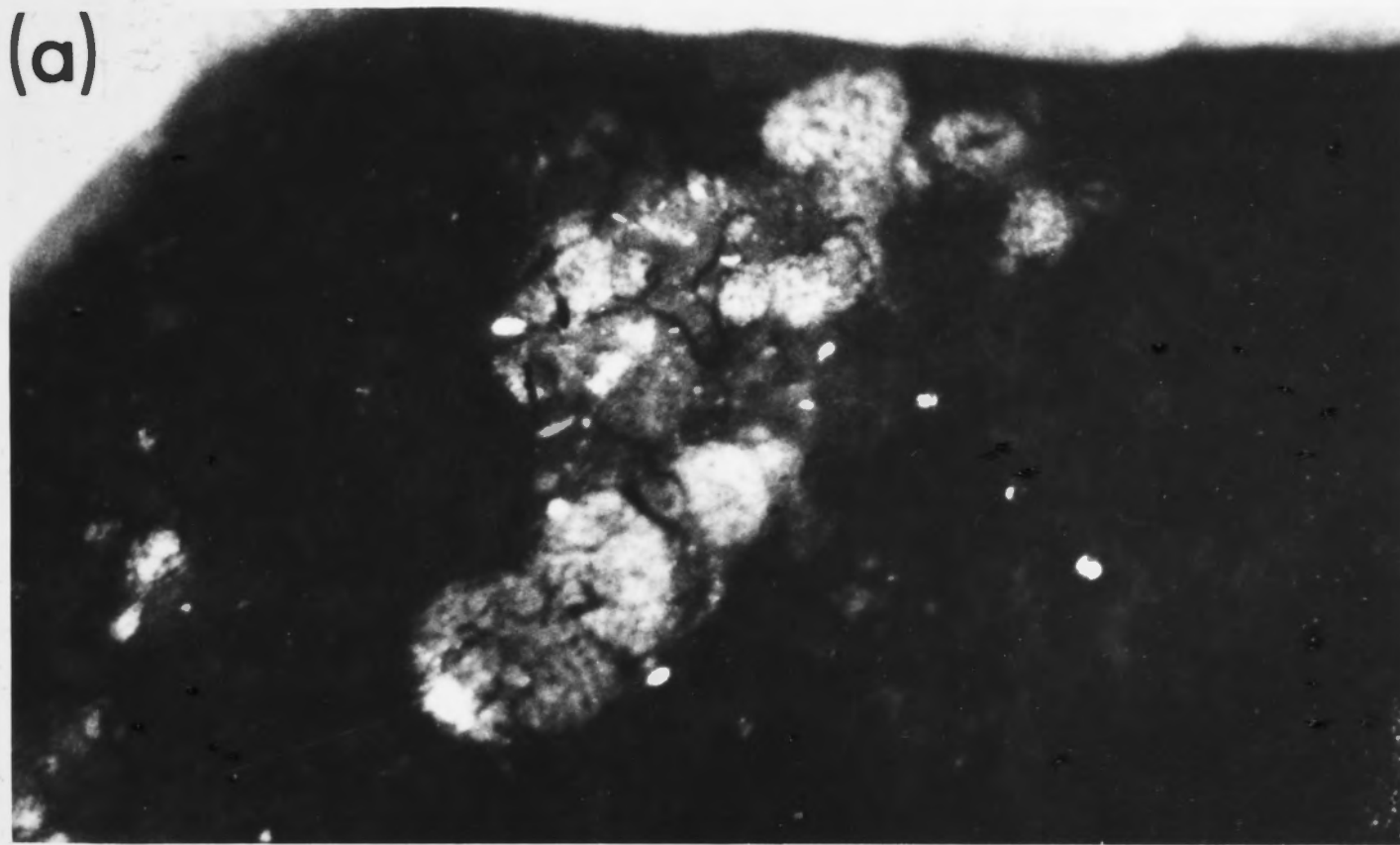


Figure 5.8 (a) Macroscopic appearance of BALB/c proislets 12 weeks after allotransplantation under the kidney capsule of a CBA recipient mouse. Note prominent revascularization and islet development. Magnification, x 152. (b) Histological section through the proislet allograft shown above (a). Note the well differentiated islet tissue and presence of some mononuclear cells which do not invade the islet tissue. Haematoxylin and eosin, x 412.

At 2 weeks post-transplantation, the survival of proislet allografts was significantly better than for uncultured and 10 day-cultured foetal pancreas allografts ($P = 0.0090$; $P = 0.0009$, respectively). All uncultured and 10 day-cultured foetal pancreas allografts were acutely rejected (Table 5.3). The histological appearance of these grafts was described in Chapter 4 (Section 4.2.1; Section 4.2.4, respectively).

These findings demonstrate that in contrast to uncultured and 10 day-cultured foetal pancreas allografts which are rapidly rejected, a proportion of proislet allografts show prolonged survival following transplantation across the same major histocompatibility barrier. The data indicate that isolated foetal mouse proislets are substantially less immunogenic than uncultured and 10 day-cultured foetal pancreas tissue.

5.3 DISCUSSION

Foetal islets have previously been isolated from rat foetal pancreas by the following procedures: collagenase digestion (Heinze and Steinke, 1971), collagenase digestion in combination with organ culture (Hellerström *et al.*, 1979), and by organ culture alone (Hegre *et al.*, 1981). Using a digestion procedure based on the method of Hellerström *et al.* (1979), we were unable to isolate foetal mouse islets. Instead, we isolated proislets from the foetal mouse pancreas. Unlike isolated foetal rat islets, isolated mouse proislets appear to represent islet precursors which continue to develop into fully differentiated islets with granulated β cells only after transplantation. One possible explanation for the difference observed between these studies is the gestation age

of the donor fetuses. In our study foetal pancreases were taken at an earlier age (17 days of gestation); rat fetuses at 21-21.5 days of gestation were used as donors of foetal islets (Heinze and Steinke, 1971; Hellerström *et al.*, 1979; Hegre *et al.*, 1981). Hence in our donor tissue, islet development was likely to be less advanced. Our histological evidence for the isolation of foetal islet precursors is supported by the finding that isografts of proislets can reverse experimentally-induced diabetes in recipient mice.

The variation observed in the function of proislet isografts is likely to be related to the variability in the yield of proislets isolated from different digests. The diabetic mice which became normoglycaemic within 40 days following isotransplantation received proislets derived from the same digest. The remaining transplanted mice each received different preparations of proislets. Thus it is possible that, with these mice, the grafts contained fewer proislets. In two of these recipients, normoglycaemia was not established until approximately 200-300 days after transplantation. This considerable delay in the appearance of graft function resembles the functional response of 20 day-cultured foetal pancreas isografts (Section 3.2.6). Together these findings illustrate the growth potential of primitive foetal islet tissue. Indeed, the presence of duct epithelium in some proislets and the administration of insulin to transplant recipients may influence the growth capacity of foetal islet precursor tissue following transplantation (see Section 3.3). It is likely that in the one transplant recipient which remained hyperglycaemic, insufficient numbers of proislets were grafted. Nevertheless, we cannot exclude

the possibility that endocrine function may have continued to develop if this graft had remained *in situ*.

Hegre and colleagues (1979) have reported that isografts of dispersed foetal rat pancreas (17.5 days of gestation) do not function as efficiently as whole foetal pancreas isografts, in reversing diabetes. For dispersed preparations, foetal pancreases were minced and then subjected to enzymatic and mechanical dissociation; the tissue was promptly transplanted without culture. To explain this difference, they suggested that some foetal islet tissue was lost or damaged during the dissociation procedure. However, it should be noted that the different site of transplantation used for the dispersed tissue may also have influenced the functional response of these grafts. Other groups working with neonatal rat pancreas have reported evidence for a significant loss of islet tissue following mincing of the tissue and collagenase digestion (Leonard *et al.*, 1973; Matas *et al.*, 1976). Matas and co-workers (1976) described the recovery of only 27% of the original tissue insulin after such treatment. On the basis of these reports, it is possible that in our study precursor islet tissue was damaged during the isolation procedure. The quantity of viable tissue lost in this manner could subsequently contribute to variation in proislet yield.

A significant finding which emerged from this study was that isolated foetal mouse proislets are not highly immunogenic and are substantially less immunogenic than uncultured and 10 day-cultured foetal pancreas. In contrast to uncultured and 10 day-cultured foetal pancreas allografts which were acutely rejected, a proportion of proislet

allografts showed evidence of prolonged survival following transplantation across the same major histocompatibility barrier. It is apparent, therefore, that the greater immunogenicity of foetal pancreas, compared to adult islets (Section 4.2.3), is not attributable to the intrinsic immunogenicity of foetal islet precursor tissue *per se*, but to some other component of the foetal pancreas. This finding is consistent with our earlier proposal that the immunogenicity of foetal mouse pancreas is probably due to the mass of primitive lymphoid tissue which is found associated with foetal pancreas explants (see Section 4.3). This lymphoid component represents an appendage of passenger leukocytes which is co-transplanted. Although organ culture of foetal pancreas in a high oxygen gas phase substantially damages this lymphoid tissue, residual lymphoid elements are probably responsible for stimulating the acute rejection of 10 day-cultured foetal pancreas allografts (see Section 4.3). Thus, the isolation of proislets appears to represent an effective method for the separation of precursor islet tissue from mouse foetal pancreas and associated lymphoid components.

The isolation of foetal proislets represents a major advance in the methodology of reducing the immunogenicity of foetal pancreas by treatment *in vitro* before transplantation. However, it is evident that the capacity to stimulate allograft rejection has not been completely obliterated. It is likely that short-term organ culture of clusters of proislets in a high oxygen gas phase would be effective in removing residual tissue immunogenicity. Faustman *et al.* (1981) have recently shown that treatment of uncultured

isolated adult mouse islets with antiserum directed against donor-specific class II alloantigens, together with complement, prior to transplantation can indefinitely prolong allograft survival. This finding supports our postulate that passenger leukocytes and not transplantation antigens carried on graft parenchymal cells, are responsible for stimulating allograft rejection (Section 1.6.1; Section 7.1). This procedure therefore represents an alternative approach to the elimination of residual proislet immunogenicity.

5.4 SUMMARY

In this chapter we describe a procedure for isolating proislets from mouse foetal pancreas. Histologically, isolated proislets are composed of undifferentiated tissue which does not stain with aldehyde fuchsin. Following isotransplantation proislets develop into well differentiated islets with β cell granulation. Thus, proislets appear to represent foetal precursor islet tissue.

Isografts consisting of proislets harvested from 8 foetal mouse pancreases can reverse Streptozotocin-induced diabetes in recipient mice.

Isolated proislets are weakly immunogenic and therefore differ significantly from the high immunogenicity of uncultured and 10 day-cultured foetal mouse pancreas. In contrast to uncultured and 10 day-cultured foetal pancreas allografts which are acutely rejected, a proportion of proislet allografts show no evidence of rejection up to 12 weeks post-transplantation.

It is apparent that the isolation of proislets represents an effective method for separating precursor islet tissue from foetal pancreas and its associated lymphoid component.

CHAPTER 6

A COMPARATIVE STUDY OF ALLOGRAFT IMMUNOGENICITY

ACROSS A MINOR HISTOCOMPATIBILITY BARRIER

6.1 INTRODUCTION

It is evident from the preceding chapters of this thesis that pancreatic islet grafts obtained from different sources or prepared for transplantation by different procedures show a marked disparity in immunogenic strength; the foetal mouse pancreas is much more highly immunogenic than either isolated adult islets or isolated foetal proislets when grafted across the same major histocompatibility barrier. We have attributed the high immunogenicity of foetal pancreas to the primitive lymphoid tissue which contaminates foetal pancreas explants (Chapter 4). On this basis, we propose that tissue-specific effects could result from differences in the quantity of passenger leukocytes carried within the transplant tissue.

The concept of tissue-specific differences in immunogenicity arose in the 1950s from comparative studies of the survival of various tissue grafts. In particular, rat ovary (Billingham and Parkes, 1955) and parathyroid (Russell and Gittes, 1959) were shown to survive for much longer periods than skin. However, the use of only partially inbred rat strains left these findings open to criticism. Subsequently, Calne and colleagues (1969) demonstrated that skin and kidney allografts were substantially more immunogenic than liver allografts transplanted between genetically undefined pig populations. Using inbred mouse strains which differed at a point mutation in the H-2 complex, Isakov *et al.* (1979) showed that heart and thyroid allografts were much less immunogenic than corresponding skin grafts. The prolonged survival of liver allografts in pigs and allogeneic heart and thyroid tissues in mice was explained by their capacity to induce the production of enhancing antibodies (Calne *et al.*, 1969) and by

the absence of donor-specific class II alloantigens (Isakov *et al.*, 1979) respectively.

Early reports by Billingham and Silvers (1964) suggested that donor age was an important factor in determining allograft survival. They demonstrated that foetal skin allotransplanted to Syrian hamsters survived longer than adult skin. On this basis, the authors suggested that foetal tissues were immunologically "inferior" to corresponding adult grafts. Similarly, Heslop (1969) reported that foetal and neonatal skin allografts survived slightly longer than adult skin grafts transplanted across the same major histocompatibility barrier. Indeed these findings probably influenced the one-time popular prediction that foetal pancreas would be less immunogenic than adult islet tissue (Brown *et al.*, 1976; Garvey *et al.*, 1979a; Mullen and Shintaku, 1980; Valente *et al.*, 1980; Groth *et al.*, 1980). We have demonstrated that this is not the case. The foetal mouse pancreas is highly immunogenic, much more immunogenic than isolated adult islets (Chapter 4) and isolated foetal proislets (Chapter 5). Thus, donor age *per se* is not a determining factor in the survival of pancreatic islet tissue allografts.

Other studies have indicated that tissue-specific differences in immunogenicity are most pronounced in the case of transplantation across "weak" or minor histocompatibility barriers (Section 1.3; Barker and Billingham, 1970). Studies by Linder (1961) demonstrated that mouse ovary was much less immunogenic than skin allografts transplanted across the same minor histocompatibility barrier; whereas ovary allografts

survived indefinitely (> 150 days), skin allografts were promptly rejected in 21-46 days. Similarly, anastomosed, auxiliary neonatal rat hearts (Barker and Billingham, 1971; Warren *et al.*, 1973), adult rat kidney (White and Hildemann, 1968) and parathyroid (Naji and Barker, 1976) have been shown in some minor histoincompatible strain combinations to survive indefinitely; in contrast, skin allografts were acutely rejected. This apparent dichotomy in tissue immunogenicity was initially attributed to differences in the vulnerability to immune processes and/or to differences in tissue antigenicity (White and Hildemann, 1968). However, subsequent investigations revealed that the lymphatic drainage of the transplant site (Naji and Barker, 1976) and the vascular integrity of the graft tissue (Warren *et al.*, 1973) can play important roles in determining allograft survival. Nevertheless, a number of tissue-specific antigens have been demonstrated (Snell, 1971). In particular, reports of skin-specific differentiation (Sk) antigens (Boyse *et al.*, 1970; Scheid *et al.*, 1972) have rendered it necessary to distinguish between tissue-specific antigenicity and immunogenicity when carrying out comparative studies of tissue allograft survival.

An effect of donor age on skin graft survival has also been demonstrated following transplantation across minor histocompatibility barriers in both mice (Hašková and Hinzová, 1966; Silvers, 1968; Wachtel and Silvers, 1971) and rats (Heslop, 1969). In particular, it has been shown that in contrast to the acute rejection of adult skin grafts, neonatal skin allografts often survived indefinitely (> 100 days). This prolonged survival has been attributed to the proliferative

capacity of perinatal tissues and their peculiar ability to induce tolerance (Hašková and Hinzová, 1966; Silvers, 1968; Heslop, 1969; Wachtel and Silvers, 1971).

In this chapter, we test the hypothesis that tissue-specific differences in immunogenicity are related to the extent of contamination of transplant tissue by passenger leukocytes and fixed lymphoreticular elements. We examine the relative immunogenicity of murine foetal pancreas, isolated adult islets and isolated foetal proislets transplanted across the same minor histocompatibility barrier. In addition, the comparative study is expanded to include allografts of mouse thyroid and skin carrying the same non-H-2 histocompatibility differences. We have selected the BALB/c (donor) and DBA/2 (recipient) strain combination for our investigation. These mouse strains are H-2 compatible ($H-2^d$) but differ in at least four known non-H-2 histocompatibility loci: H-1, H-7, H-8 and H-13 (Graff, 1970).

6.2 RESULTS

6.2.1 Transplantation of pancreatic islet tissue across a minor histocompatibility barrier

Table 6.1 shows that allografts of a single uncultured foetal pancreas, transplanted across a minor histocompatibility difference, were acutely rejected. By 4 weeks post-transplantation, all 6 grafts consisted of scar tissue with some light mononuclear cell infiltration; there was no evidence of pancreatic islet tissue remaining. In contrast, six of eleven allografts composed of six 20 day-cultured foetal pancreas segments showed no signs of rejection at 4

TABLE 6.1

Survival of BALB/c pancreatic islet allografts following
transplantation to DBA/2 recipients

Allograft	Treatment	Total No. grafts ^a	No. grafts with histological score ^b					% grafts surviving ^c
			++++	+++	++	+	0	
Foetal pancreas	Uncultured	6	- ^{d,e,f}	-	-	6	-	0
	20 day- cultured	11	6 ^d	2	1	1	1	55
Proislets	-	11	10 ^e	-	-	-	1	91
Isolated adult islets	Uncultured	5	4 ^f	-	1	-	-	80
	7 day- cultured	6	6	-	-	-	-	100

a. Grafts were examined at 4 weeks post-transplantation.

b. Histological score key:

++++ Intact islets without mononuclear cell infiltration at the graft site.

+++ Intact islets with mononuclear cell infiltration at the graft site but no infiltration into islets.

++ Mononuclear cell infiltration and damaged islet tissue present.

+ Mononuclear cell infiltration and scar tissue.

0 Fibrotic scar tissue or necrotic tissue without mononuclear cell infiltration.

c. Criteria for graft survival were the presence of intact islets and the absence of mononuclear cell infiltration at the graft site.

d. $P = 0.0746$ (Exact probability test; Swinscow, 1980b).

e. $P = 0.0012$ (Exact probability test; Swinscow, 1980b).

f. $P = 0.0303$ (Exact probability test; Swinscow, 1980b).

weeks after transplantation. These grafts contained intact, well differentiated islets and some fibrotic connective tissue. There was no evidence of mononuclear cell infiltration. Two of the remaining grafts showed well developed islets in the presence of a light mononuclear cell infiltrate which did not penetrate the islet tissue. One graft consisted mainly of fibrotic connective tissue and a few islet remnants. It is likely that the lack of islet survival in this instance was due to technical failure (Section 4.2.4). The remaining grafts showed obvious signs of on-going or complete rejection. Although statistical tests showed that the survival (as defined in Table 6.1) of 20 day-cultured allografts was not significantly different, at the 5% level, to uncultured foetal pancreas allografts ($P = 0.0746$), analysis of the range of histological scores revealed that there was a significant effect of organ culture on allograft integrity; eight of eleven 20 day-cultured allografts scored better than the total group of six uncultured allografts ($P = 0.0045$; Exact probability test; Swinscow, 1980b).

Allografts of foetal proislets harvested from two foetal pancreases showed no evidence of rejection at 4 weeks. Ten of eleven grafts contained intact, well developed islets and no mononuclear cell infiltration at the graft site. The remaining graft showed only remnants of islet tissue, but no cellular infiltrate. It is possible, therefore, that this graft was a technical failure. From a statistical standpoint, the survival of proislet allografts was significantly better than uncultured foetal pancreas allografts ($P = 0.0012$).

At 4 weeks post-transplantation, four of five allografts of a single cluster of uncultured isolated adult islets (Section 2.11) consisted of a mass of intact islet tissue without mononuclear cell infiltration. The remaining graft showed signs of rejection. There was no significant difference in the survival of uncultured and 7 day-cultured allografts of isolated adult islets; each of six allografts of a 7 day-cultured cluster of adult islets (Section 2.6) consisted of intact islet tissue and showed no evidence of rejection.

Statistical analysis showed that the survival of allografts of uncultured isolated adult islets was significantly better than for uncultured foetal pancreas allografts ($P = 0.0303$) transplanted across the same minor histocompatibility barrier.

Thus, uncultured foetal mouse pancreas allografts carrying multiple minor histocompatibility differences were more highly immunogenic than corresponding untreated allografts of isolated adult islets. In addition, foetal proislets were significantly less immunogenic than uncultured foetal pancreas. Organ culture for 20 days in a 95% O_2 , 5% CO_2 gas phase significantly improved the histological integrity of foetal pancreas allografts transplanted across the same minor histocompatibility barrier.

6.2.2 Survival of full-thickness orthotopic skin grafts

Table 6.2 shows that BALB/c orthotopic skin grafts were acutely rejected following transplantation across a minor histocompatibility barrier to DBA/2 recipient mice. Seven of nine skin allografts were rejected in 11-13 days. One of the remaining transplant recipients died on day 11 from an

anesthetic overdose. Histological examination of the skin graft showed necrosis of the graft epithelium and the presence of a moderate to heavy mononuclear cell infiltrate in the underlying dermis (Fig. 6.1). These findings suggested that the allograft was in the process of being rejected. The remaining skin graft was discarded.

TABLE 6.2

Survival of BALB/c full-thickness orthotopic skin grafts

Recipient strain	Total No. grafts	Graft survival ^a (days)	% grafts surviving
DBA/2	9	11, >11 ^b , 13, 13, 13, 13, 13, 13, >13 ^c	0
BALB/c	5	>435, >477, >477, >477, >477	100

a. Skin graft survival was assessed by macroscopic appearance. Criteria for endpoint of graft survival were complete dehydration and scabbing of the epidermis.

b, c See text (Section 6.2.2).

anaesthetic overdose. Histological examination of the skin graft showed necrosis of the graft epithelium and the presence of a moderate to heavy mononuclear cell infiltrate in the underlying dermis (Fig. 6.1). These findings suggested that the allograft was in the process of being rejected. The remaining skin graft showed signs of partial dehydration on day 13. At 39 days after transplantation, only a small scab remained at the graft site. This finding indicated that the graft had been rejected at ≤ 39 days after transplantation. All five BALB/c skin isografts survived indefinitely (see Section 2.14).

Thus, the non-H-2 incompatible skin allografts were highly immunogenic. This finding confirms the early report by Prehn and Main (1954) that BALB/c skin grafts were readily rejected by DBA/2 recipient mice. They observed graft rejection by 18-42 days after transplantation. The discrepancy in the graft survival times obtained between the latter study and our own findings can probably be attributed to the different criteria used for the endpoint of graft survival (Table 6.2). Prehn and Main (1954) chose total dislodgement of the escharified graft from the host bed as their criterion for rejection.

6.2.3 Function of thyroid allografts following transplantation to naive and sensitized recipients

We set out to determine whether tissue-specific differences in immunogenicity following transplantation across a minor histocompatibility barrier were related to differences in tissue antigenicity.

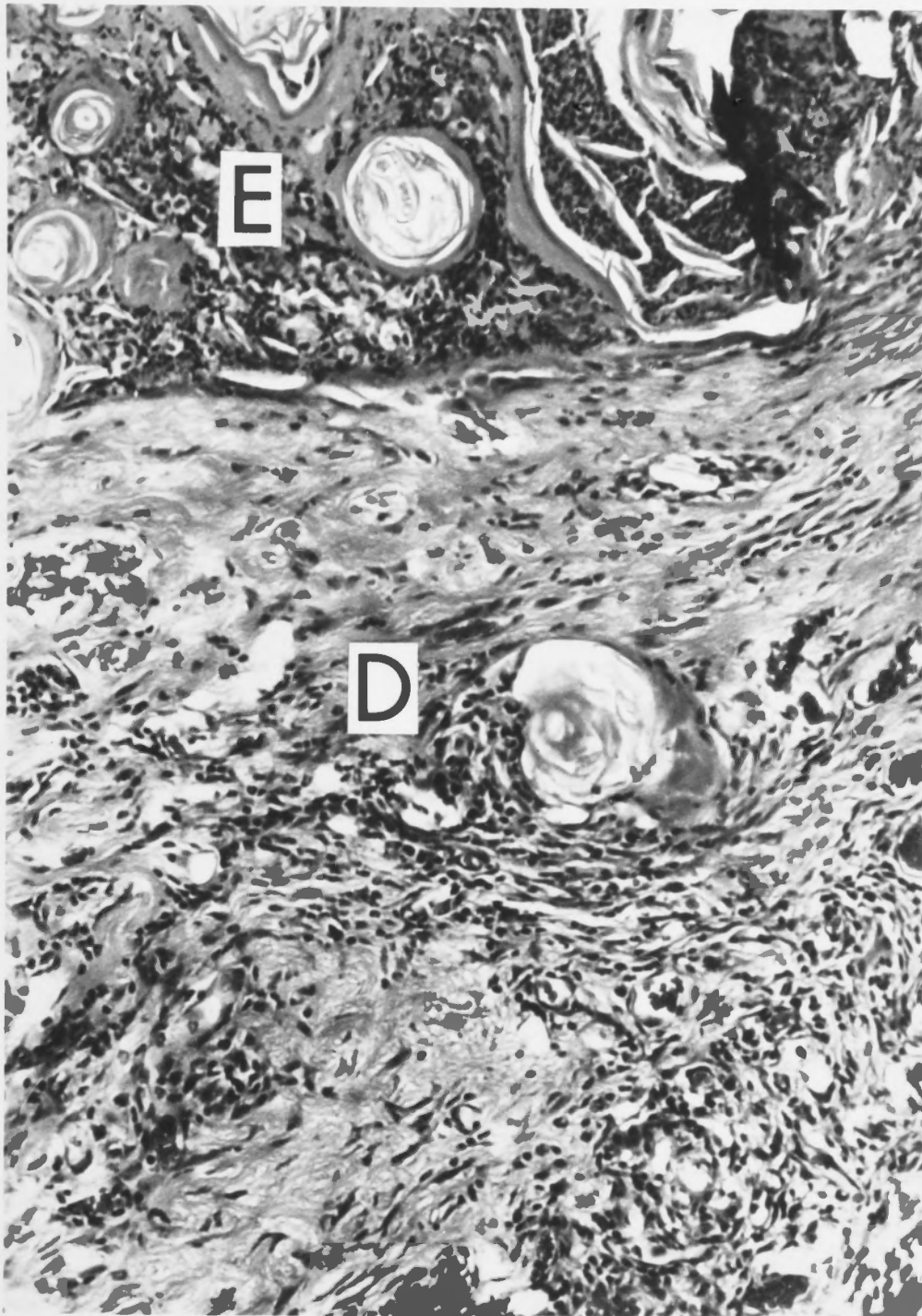


Figure 6.1 Histological appearance of an orthotopic BALB/c skin allograft, 13 days after transplantation to a DBA/2 recipient mouse. Note necrotic epidermis (E) and mononuclear cell infiltration in dermis (D). Haematoxylin and eosin, x 172.

Table 6.3 shows the proportion of BALB/c thyroid allografts which functioned following transplantation to naive DBA/2 recipient mice. Twenty-five of twenty-seven uncultured thyroid allografts functioned for ≥ 45 days (Section 2.21). The remaining two grafts failed to concentrate ^{125}I on two consecutive occasions. At 32 days after transplantation, the two mice bearing non-functional grafts were sacrificed and the thyroid transplants were taken for histological examination. Both allografts contained a heavy mononuclear cell infiltrate with some intact follicles still remaining. This histological picture indicated that both grafts were succumbing to a rejection reaction. Of the twenty-five functioning allografts, twenty-three remained functional for > 100 days (Fig. 6.2); the hosts of the other two functional grafts died at 55-56 days post-transplantation. Thus, good functional survival of thyroid allografts was observed without the need for conditioning of the graft tissue before transplantation. Indeed, there was no significant difference between the survival of uncultured and cultured thyroid allografts carrying the same minor histocompatibility differences. It should be noted that cultured thyroid allografts were prepared from cyclophosphamide-pretreated donors (Section 2.7). Each of nineteen 21 day-cultured thyroid allografts functioned for ≥ 45 days. Of these, eighteen grafts remained functional for > 100 days; the host carrying the remaining graft died at 74 days post-transplantation. Thus, there was no evidence of rejection of thyroid allografts which had been prepared from cyclophosphamide-pretreated donors and cultured for 21 days in a 95% O_2 , 5% CO_2 gas phase before transplantation to naive DBA/2 recipient mice (Fig. 6.3(a)).

TABLE 6.3

Function of BALB/c thyroid allografts transplanted
to thyroidectomized DBA/2 recipients

Treatment of thyroid tissue	Pretreatment of recipient	Total No. grafts	No. grafts showing function ^a	% functioning grafts
Uncultured	-	27	25 ^c	93
21 day- cultured	-	19	19 ^{c,d}	100
21 day- cultured	BALB/c skin ^b graft	6	0 ^d	0

a. Criterion for graft function was sequestration of ^{125}I for ≥ 45 days after transplantation (Section 2.21; Section 2.20).

b. Each DBA/2 mouse received a BALB/c skin allograft at 80-87 days prior to thyroid transplantation.

c. $P = 0.6783$ (Exact probability test; Swinscow, 1980b).

d. $P = 0.0000$ (Exact probability test; Swinscow, 1980b).

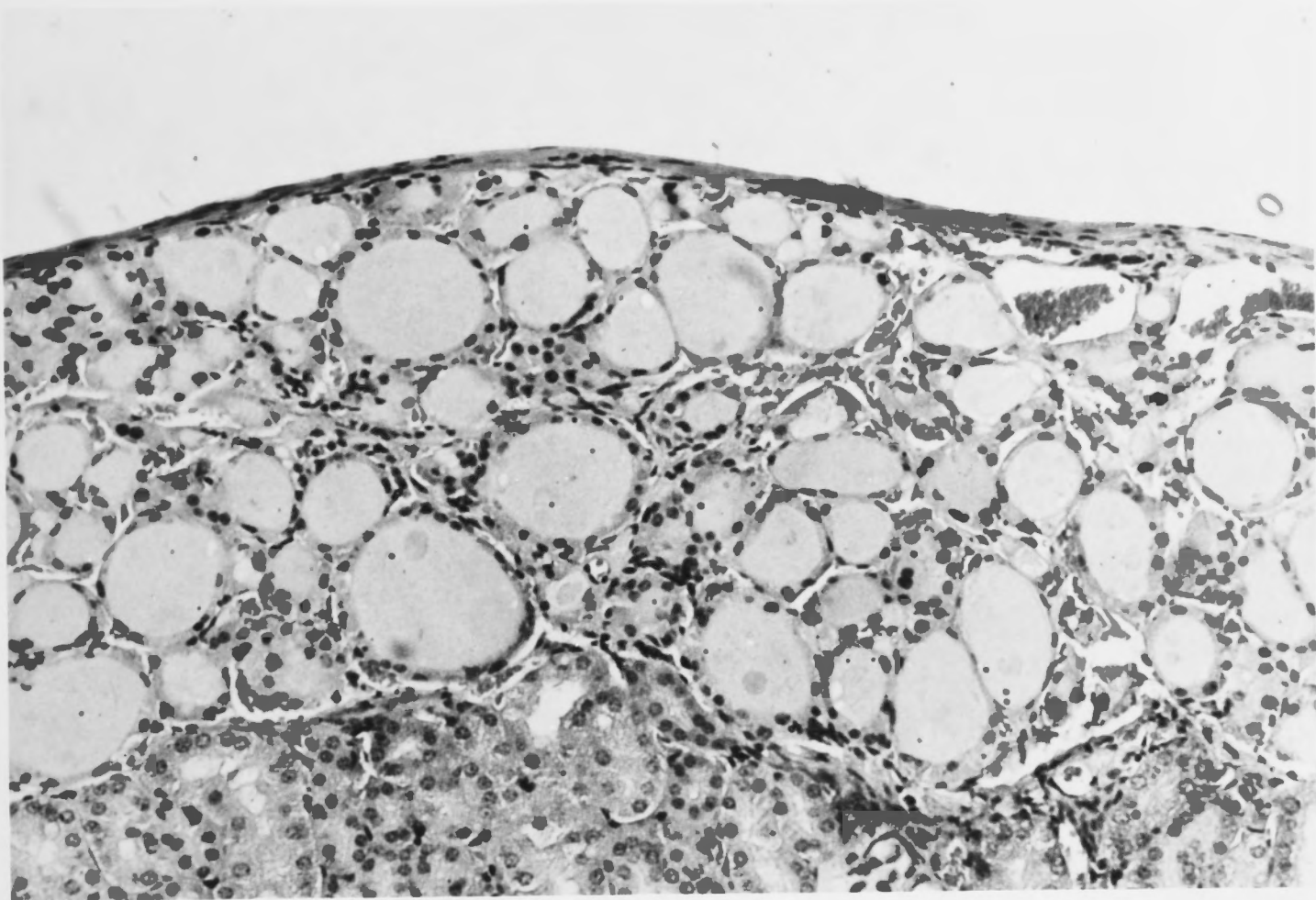


Figure 6.2 Allograft of uncultured BALB/c thyroid at 328 days after transplantation under the kidney capsule of a DBA/2 recipient mouse. Note presence of intact thyroid follicles and lack of a rejection response. Haematoxylin and eosin, x 172.

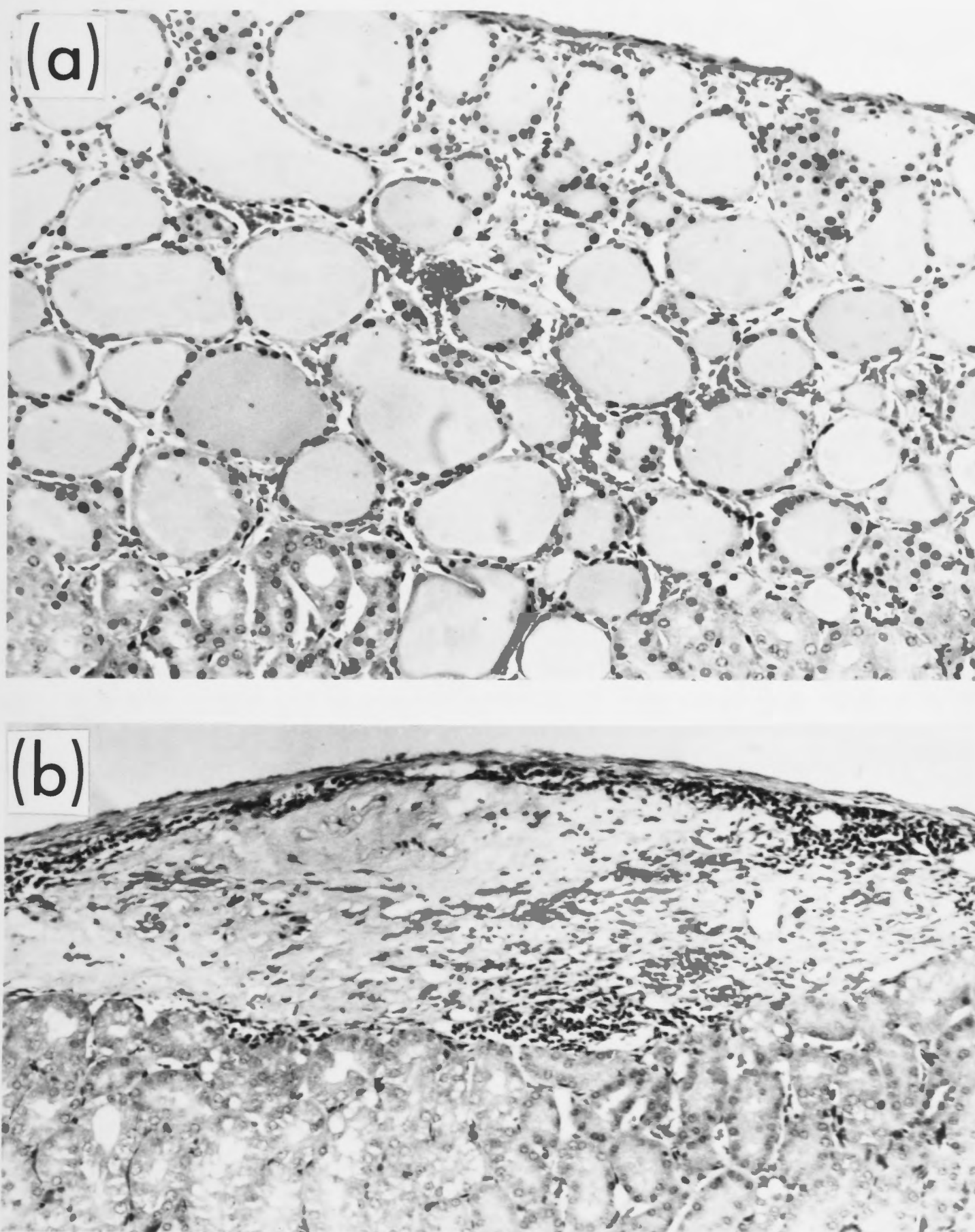


Figure 6.3 (a) Allograft of 21 day-cultured BALB/c thyroid at 348 days after transplantation beneath the kidney capsule of a naive DBA/2 recipient mouse. Note intact thyroid follicles and absence of mononuclear cell infiltration. Haematoxylin and eosin, x 171. (b) Allograft of 21 day-cultured BALB/c thyroid at 24 days after transplantation to a DBA/2 recipient mouse presensitized with a BALB/c skin graft, 80 days before thyroid transplantation. Note scar tissue, mononuclear cells and lack of intact thyroid tissue remaining. Haematoxylin and eosin, x 159.

Table 6.3 also shows that 21 day-cultured BALB/c thyroid allografts were rejected following transplantation to DBA/2 recipient mice which had been presensitized by a BALB/c skin graft. The sensitized recipients were derived from the experiment detailed in Table 6.2. None of the six 21 day-cultured thyroid allografts transplanted to sensitized recipients showed evidence of graft function. At 24-32 days post-transplantation, the transplant recipients were sacrificed and the thyroid allografts were examined histologically. Five of the six grafts showed only scar tissue, a light mononuclear cell infiltrate and, occasionally, some haemorrhage (Fig. 6.3(b)). The remaining graft contained a moderate to heavy mononuclear cell infiltrate and several residual thyroid follicles. Thus, the cultured thyroid allografts underwent acute rejection following transplantation to sensitized recipients.

In contrast to the high immunogenicity of non-H-2 histoincompatible skin allografts, thyroid tissue is weakly immunogenic when transplanted across the same minor histocompatibility barrier. Nevertheless, the acute rejection of 21 day-cultured thyroid allografts by recipients sensitized by a skin graft of the same donor specificity illustrates that the disparity in the survival of skin and thyroid allografts is not due to the lack of recognizable minor alloantigens on thyroid tissue or to the presence of tissue-specific antigens on skin.

6.3 DISCUSSION

This work was carried out to analyse the basis for tissue-specific differences in immunogenicity and to determine whether the phenomenon is related to the quantity of passenger leukocytes and fixed lymphoreticular cells in the transplant tissue.

The data presented in this chapter demonstrate that foetal mouse pancreas is highly immunogenic even when allografted across a non-H-2 histocompatibility barrier. This finding is consistent with reports that foetal rat pancreas allografts are acutely rejected following transplantation across minor histocompatibility differences (Spence *et al.*, 1979; Mullen and Shintaku, 1980). Furthermore, we found that like foetal pancreas tissue, orthotopic skin allografts in the same donor/recipient mouse strain combination were acutely rejected. In contrast to the high immunogenicity of foetal pancreas and skin, 80% of uncultured allografts of islets from the adult pancreas and 93% of untreated thyroid grafts transplanted across the same minor histocompatibility barrier showed no evidence of rejection. Thus, mouse thyroid and isolated adult islets are weakly immunogenic in this transplant system.

Other groups working with rats (Reckard and Barker, 1973) and mice (Frangipane *et al.*, 1977; Naji *et al.*, 1981a) have reported that with some minor histoincompatible strain combinations, allografts of isolated adult islets are rapidly rejected. Furthermore, treatment of transplant recipients with antilymphocyte serum (ALS) in most instances only temporarily prolonged islet allograft survival (Reckard and

Barker, 1973; Frangipane *et al.*, 1977). Delayed rejection of minor histoincompatible allografts of adult rat islets has also been observed (Ziegler *et al.*, 1975). The discrepancies between these studies and our own may be related to the differences in minor immunogenetic barriers and/or to the method of islet isolation. It is evident from these reports that care was not taken to remove small contaminating lymph nodes from the preparations of isolated islets (Section 4.3). It is our hypothesis that the presence of such lymphoid elements of donor origin renders islet transplants highly immunogenic. Consequently, we attribute the weak immunogenicity of our preparations of isolated adult mouse islets to the removal of small lymph node contaminants by careful hand-picking of the individual islets.

An important finding in this chapter was that treatment of foetal mouse pancreas *in vitro* before allotransplantation across multiple non-H-2 histocompatibility differences can influence allograft integrity. Both organ culture in a 95% O₂, 5% CO₂ gas phase and the preparation of proislets were effective in reducing the immunogenicity of foetal pancreas tissue. In contrast, Naji and colleagues (1981b) failed to observe a positive effect of long-term organ culture, in a high oxygen gas phase, on the functional survival of rat parathyroid allografted across minor histocompatibility differences; the same culture system markedly prolonged the survival of MHC-incompatible parathyroid allografts. To explain this failure, they proposed that the foreign minor antigens of the graft were processed by host macrophages, presumably via class I structures. Presentation of the minor alloantigens on host macrophages then activated a

graft-specific T cell response. Implicit in this proposal was the idea that donor passenger leukocytes were not involved in the stimulation or effector phase of the rejection mechanism. Indeed, these ideas are consistent with both the failure of *in vitro* studies to demonstrate primary MLC reactivity when stimulator and responder cell populations are H-2 compatible but non-H-2 disparate (Bevan, 1976a; Wettstein and Frelinger, 1977) and the ability to overcome this lack of response by *in vivo* priming for "secondary" MLCs (Bevan, 1975, 1976a; Wettstein *et al.*, 1977). Bevan (1976a) attributed the latter phenomenon to "cross-priming" and suggested that the mechanism involved processing of donor minor antigens by host macrophages (Bevan, 1976b). In accordance with this premise, H-2D or H-2K plus I region compatibility between the target and host responding cell populations was required for the demonstration of specific target lysis *in vitro* (Bevan, 1976b). Similarly, the induction of GVH disease in minor histoincompatible systems (Sprent and Korngold, 1981) and the generation of "secondary" MLC responses to individual minor alloantigens following *in vivo* immunization (Gordon *et al.*, 1976; Wettstein and Frelinger, 1980) have indicated that the alloreactivity of T cells to foreign minor antigens is restricted by class I antigens.

Our data do not agree with the interpretations of these other groups and, furthermore, raise the possibility that foreign non-H-2 antigens may be processed via class II structures. This proposal is based on the following observations. It has been demonstrated that class I antigens are expressed on both cultured and uncultured mouse thyroid (Parr *et al.*, 1980a) and on uncultured isolated adult mouse

islets (Faustman *et al.*, 1980; Parr *et al.*, 1982). Consequently, if processing of graft non-H-2 alloantigens by host antigen presenting cells occurs via class I structures, then mouse thyroid allografts and allografts of isolated adult mouse islets would be susceptible targets for T cell-mediated damage. However, the long-term survival of cultured thyroid allografts as well as of the majority of uncultured thyroid allografts and allografts of uncultured adult islets, argues against the premise that minor antigens are processed through class I structures. Indeed, evidence for processing of foreign antigens via class II structures has been reported for DTH responses to fowl gamma globulin (Miller *et al.*, 1976; dinitrofluorobenzene (Miller *et al.*, 1976) and the H-Y minor alloantigen (Liew and Simpson, 1980), as well as for T cell-dependent antibody responses to sheep red blood cells (Sprent and Korngold, 1981). Furthermore, the *in vivo* capacity of UV-inactivated influenza virus to activate a primary helper T cell-dependent antibody response (Braciale and Yap, 1978) and to generate Lyt-1^+2^- T cells capable of mediating DTH reactions (Leung *et al.*, 1980) suggests that viral antigens may also be processed through class II antigens on host antigen-presenting cells.

If foreign minor antigens released from tissue allografts bearing only non-H-2 histocompatibility differences are processed via class II structures on host antigen-presenting cells then the activity of sensitized T cells would be restricted by the requirement for simultaneous recognition of class II antigens. Thus, graft-specific T cell responses would be generated only for those grafts which also carry class II antigens. As mentioned previously, class II antigens

are characteristically expressed on cells of lymphoreticular origin and are absent from tissue parenchymal cells (Section 1.3). Consequently, only those tissue allografts contaminated with lymphoid elements of donor origin would be susceptible to T cell-mediated damage. It is suggested here that lymphokine(s) released by activated T cells following recognition of both minor alloantigens and class II structures within the graft may initiate inflammatory reactions which could lead to local non-specific tissue damage. Such responses could result from the activation and proliferation of responsive blood cells of donor origin and/or from the recruitment of non-specific host inflammatory cells (Section 1.6.1). We propose that this rationale accounts for the high immunogenicity of minor histoincompatible uncultured foetal pancreas and skin allografts since a distinctive property of these tissues is the presence of an associated or intrinsic lymphoreticular component, respectively. Conversely, the weak immunogenicity of isolated foetal proislets, isolated adult islets and thyroid transplanted across the same non-H-2 histocompatibility barrier can be explained by the lack of gross lymphoid contamination of these tissues. On this basis, the reduction in the immunogenicity of foetal pancreas tissue following organ culture and the preparation of proislets can be attributed to the extent to which donor lymphoid tissue is damaged prior to allotransplantation.

Further support for our postulate is provided by our demonstration that tissue-specific differences in immunogenicity were not due to the expression of tissue-specific antigens or to the lack of recognizable minor alloantigens. The similarity in the weak immunogenicity of isolated foetal

proislets and isolated adult islets suggested that the immunogenic potency of whole foetal pancreas allografts was not attributable to foetal antigens. Likewise, the acute rejection of 21 day-cultured BALB/c thyroid allografts by DBA/2 mice presensitized by a BALB/c skin graft eliminated a role for tissue-specific antigen(s) as the factor determining the high immunogenicity of skin allografts and demonstrated that the cultured thyroid tissue carried recognizable minor allo-antigens. Nevertheless, the rejection of the cultured thyroid tissue in this situation leads us to suggest that the process of sensitization by the primary skin graft and/or the mechanism of skin graft rejection may have led to an "adjuvant-like" effect on the activation of host T cells carrying receptors for both donor-specific minor alloantigens and class I structures. However, this explanation does not resolve the paradox between our data and the reports by Silvers' group (Naji *et al.*, 1981b; Silvers *et al.*, 1982) on the effect of organ culture on facilitating the transplantation of graft tissue across a minor histocompatibility barrier.

Although the phenomenon of tissue-specific differences in immunogenicity following transplantation across minor histocompatibility differences has been recognized for some time (Section 6.1), analysis of the mechanism underlying these disparities is still in its infancy. The data presented in this chapter, together with the present literature, reveal the paucity of our understanding of these immune processes. It is obvious that these experimental systems require further analysis in future research. In relation to our own postulate, it would be worthwhile to graft uncultured foetal

pancreas and 7 day-cultured isolated adult islets simultaneously to the same host. The rejection of both types of donor tissue would support the concept of an adjuvant-like effect. Other studies in our laboratory have shown a difference in the susceptibility to rejection, across a major histocompatibility barrier, of long-established allografts of isolated adult islets and 10 day-cultured foetal pancreas tissue of the same donor specificity freshly allotransplanted to the same host; whereas the foetal pancreas tissue was acutely rejected, the isolated adult islets remained intact (M. Agostino, personal communication). In the latter situation, the absence of an adjuvant-like effect may be due to the induction of tolerance to donor-specific class I antigens (Section 7.1).

In any case, the study detailed in this chapter illustrates that modulation of the immunogenicity of foetal pancreas tissue carrying only minor histocompatibility allo-antigens can be achieved at the level of pretreating the donor tissue *in vitro*. This approach eliminates the need for modifying the immune status of the host by immunosuppressive agents (Mullen, 1980) or by the induction of donor-specific tolerance (Spence *et al.*, 1979).

6.4 SUMMARY

In this chapter we demonstrated tissue-specific differences in immunogenicity following allotransplantation across the same minor histocompatibility barrier. In contrast to the high immunogenicity of foetal pancreas and skin, isolated adult islets, foetal proislets and thyroid were weakly immunogenic.

Reduction in the immuogenicity of foetal mouse pancreas tissue was achieved by the preparation of foetal proislets and by organ culture of foetal pancreas segments for 20 days in a high oxygen gas phase. Thus, the immunogenic status of minor histoincompatible foetal pancreas allografts can be modulated by treating the donor tissue *in vitro* before transplantation.

Evidence against a role for tissue-specific antigens in determining strong tissue immunogenicity was provided by:

- (a) the rejection of 21 day-cultured thyroid allografts by recipient mice presensitized by donor-specific skin; and
- (b) the much weaker immunogenicity of allografts of isolated foetal proislets compared to whole foetal pancreas allografts.

We have postulated that the immunogenic strength of minor histoincompatible tissue allografts is related to the extent to which the graft tissue is contaminated by donor lymphoreticular cells which may act as targets for T cell-mediated reactions and thereby contribute to non-specific graft destruction.

CHAPTER 7

TRANSPLANTATION IMMUNOBIOLOGY:

THE STATE OF THE ART

7.1 GENERAL DISCUSSION

The aim of this thesis was to determine whether the approach of modulating tissue immunogenicity by *in vitro* procedures was applicable to pancreatic islet tissue and, in particular, to the foetal mouse pancreas. Our study has illustrated that the immunogenicity of foetal mouse pancreas can be substantially reduced, without loss of functional integrity, by organ culture in a high oxygen gas phase and by the preparation of foetal mouse proislets. Indeed, we have demonstrated that allografts of 20 day-cultured foetal pancreas tissue, transplanted across a major histocompatibility barrier, can reverse Streptozotocin-induced diabetes in recipient mice. A comparative study of the effect of organ culture on the immunogenicity of foetal and adult islet tissue indicated that foetal mouse pancreas was much more difficult to condition for allotransplantation than islets isolated from the adult pancreas. In view of our postulate that organ culture of donor tissue facilitates allotransplantation by removing immunostimulatory passenger leukocytes (Section 1.6.3), we have attributed this disparity in immunogenicity to the extensive contamination of foetal pancreas explants with primitive lymphoid tissue and to the steps taken to avoid gross lymphoid contaminants in preparations of isolated adult islets. Furthermore, we propose that the effectiveness of organ culture and the isolation of proislets in reducing foetal pancreas immunogenicity is due to the degree to which the associated lymphoid component is removed from the primitive endocrine tissue. In the case of cultured adult islets, we have demonstrated that the reduction in allograft immunogenicity is

not attributable to loss of tissue antigenicity and that immunogenic status can be restored by the administration of donor-specific viable peritoneal cells at the time of transplantation. Our findings are consistent with the postulate that passenger leukocytes and not transplantation antigens *per se* constitute the major barrier to allotransplantation; passenger leukocytes represent the only cells of donor origin capable of activating a graft-specific T cell response (Section 6.1).

Batchelor and colleagues (1979), working with an organ transplantation model in rats, have reached a similar conclusion. They investigated the immunogenicity of long-established, passively enhanced kidney allografts following secondary transplantation to naive recipients isogeneic to the primary host. A proportion of retransplanted grafts survived indefinitely and these grafts failed to elicit normal T cell-dependent humoral and cellular immune responses, although they expressed donor alloantigens. Consequently graft alloantigen *per se* was shown to be an insufficient stimulus for T cell activation. Nevertheless, the authors suggested that the induction of T cell-independent IgM antibody responses may be sufficient in some rat strain combinations to destroy kidney graft integrity (Batchelor *et al.*, 1979; Welsh *et al.*, 1979). Such an explanation could account for earlier failures to successfully retransplant enhanced kidney allografts (Stuart *et al.*, 1971; Section 1.6.2). The finding by Welsh *et al.* (1979), that the reduction in the immunogenicity of enhanced kidney grafts was time-dependent, supported the idea that donor passenger leukocytes present in either the graft's vasculature or interstitial tissue represented the immunogenic stimulus of

normal kidney allografts (Batchelor *et al.*, 1979; Welsh *et al.*, 1979).

In support of this proposal, immunogenicity was most effectively restored to long-term enhanced kidney allografts by the administration of donor-specific dendritic cells to secondary hosts at the time of retransplantation (Lechler and Batchelor, 1982). Indeed, dendritic cells which are derived from bone marrow have been shown to be potent stimulators in primary mixed lymphocyte cultures (Steinman and Witmer, 1978). Thus, they have the capacity to provide both signals required for T cell activation. Relevant to this argument is the report by Hart and Fabre (1981) that dendritic cells are present in the interstitial connective tissue of rat kidney as well as of a number of other tissues including heart, liver, pancreas, thyroid and skin. On the basis of these findings, the dendritic cell which expresses both class I and class II antigens probably represents a potent immunostimulatory passenger leukocyte in a variety of transplant tissues. The failure of donor-specific B lymphocytes, which also carry both class I and class II alloantigens, to restore the immunogenic status of both enhanced kidney allografts (Lechler and Batchelor, 1982) and long-established islet allografts (Zitron *et al.*, 1981b) in rats is seen as additional evidence against Bach's postulate that passenger leukocytes are important in tissue immunogenicity because they provide the class II alloantigens required for the induction of cytotoxic T cells (Section 1.6.1). It should be noted that while expression of class II alloantigens does not distinguish S^+ status, cells of the lymphoreticular lineage which do express S^+ activity (Section 1.5.3) also carry class II alloantigens.

Interestingly, the rejection of established allografts was also observed following the administration of unsensitized T cells of donor origin (Zitron *et al.*, 1981b; Lechler and Batchelor, 1982). This immune response has been attributed to activation of host T cells by donor dendritic cells contaminating the inoculum (Lechler and Batchelor, 1982). Faustman and colleagues (1981) have provided conclusive evidence for a role for class II alloantigen-bearing cells in the activation of the rejection response to isolated adult mouse islets. Incubation of the adult islets with antisera directed against donor-specific class II alloantigens, together with complement, just prior to transplantation indefinitely prolonged the survival of islet allografts transplanted across a major histocompatibility barrier to nonimmunosuppressed recipients. Since class II antigens are almost exclusively expressed on lymphoid tissues (Section 1.3) and are not carried by pancreatic beta cells (Faustman *et al.*, 1980; Parr *et al.*, 1982), the effect of the antiserum treatment was attributed to the removal of passenger leukocytes. Indeed, this procedure may be useful in further reducing the immunogenicity of isolated foetal proislets before transplantation.

Taken together, these findings support our proposal that modulation of the immunogenicity of pancreatic islet tissue by organ culture and also in the case of foetal pancreas, by the isolation of proislets, is achieved by the removal of passenger leukocytes. Mandel *et al.* (1982) have confirmed our findings by demonstrating prolonged survival of allogeneic foetal mouse pancreas following long-term organ culture in a high oxygen gas phase. In addition, Lacy and colleagues (1982) have reported that organ culture for 7 days in a 95% O₂, 5% CO₂ gas

phase facilitated the xenotransplantation of isolated adult rat islets to recipient mice. Naji *et al.* (1979) have provided evidence indicating that the reduction of rat parathyroid immunogenicity by organ culture is oxygen-dependent. This finding supports the proposal that high oxygen levels are toxic for passenger leukocytes (Section 1.6.3).

An important finding which emerged from our study was that organ culture is more effective in reducing the immunogenicity of tissues which do not carry gross lymphoid components. Similarly, the marked resistance of allogeneic skin to procedures aimed at reducing the tissue's immunogenicity (Steinmuller, 1980; Emma and Jacobs, 1981; Section 1.6.2) is probably related to the intrinsic or fixed population of dendritic-like cells called the Langerhans cells (Steilein and Bergstresser, 1980). To test the extent to which these Langerhans cells contribute to skin allograft immunogenicity, Steinmuller (1981) used donor skin from chimeric "A" mice at least 100 days after reconstitution with allogeneic "B" spleen cells. At this time after reconstitution, the Langerhans cell and passenger leukocyte populations were shown to be of "B", the prospective recipient, specificity. However, following transplantation of chimeric "A" skin across a minor histocompatibility barrier to naive "B" mice, the allografts were still rejected. This finding led Steinmuller to conclude that neither passenger leukocytes nor Langerhans cells contributed to skin immunogenicity (Steinmuller, 1981). Consequently, he has turned to the concept of skin-specific antigens as a possible explanation for the high immunogenicity of this tissue (Steinmuller *et al.*, 1981, 1982). On the basis of our study of tissue-specific differences in immunogenicity

following transplantation across a minor histocompatibility barrier (Chapter 6), we suggest that Steinmuller's data can be explained by processing of the "A" type minor alloantigens by "B" type Langerhans cells present in the chimeric skin graft and by "B" type antigen-presenting cells of the transplant recipient. Indeed, Langerhans cells are recognized as the major antigen-presenting cells in contact hypersensitivity (Streilein and Bergstresser, 1980) and can present foreign antigen *in vitro* to primed isogeneic T cells (Stingl *et al.*, 1978). According to our explanation, recipient-type Langerhans cells present in the skin graft would then function not only in stimulating a graft-specific T cell response, but also as targets for T cell-mediated damage. On the basis of our data on the allotransplantability of foetal pancreas tissue across a minor histocompatibility difference, we suggest that successful allotransplantation of non-H-2 incompatible skin will require the complete elimination of both the passenger leukocyte and Langerhans cell populations before transplantation. *In vitro* procedures incorporating dissociation of skin into single cell suspensions, treatment with antiserum directed against class II-antigen bearing cells, plus complement, followed by the reaggregation of the dissociated skin cells into monolayers, may represent one approach worthy of investigation. In support of our argument, Oluwole and colleagues (1980) have demonstrated a lack of correlation between the removal of mobile passenger leukocytes from rat heart, before transplantation, and graft survival following transplantation across a minor histocompatibility barrier. They have attributed this finding to the immunogenicity of

residual, fixed interstitial cells. This explanation is supported by the identification of dendritic cells in the interstitial connective tissue of rat heart (Hart and Fabre, 1981).

Thus, it is not unreasonable to propose that tissue-specific differences in immunogenicity may well result from the aggregate load of mobile passenger leukocytes, fixed dendritic cells and associated lymphoid components. We suggest that this explanation is applicable not only to tissue allotransplantation across a major histocompatibility barrier (Chapter 4) but probably also in the case of only non-H-2 immunogenetic differences (Chapter 6). In the former situation, mobile and fixed or associated lymphoreticular cell populations would function as S^+ cells (Section 1.6.1) in activating a graft-specific T cell response; in both situations these cells may act as targets for T cell effectors and thereby contribute to non-specific graft destruction (Section 1.6.1).

There is now conclusive evidence that graft rejection is a T cell-mediated process. Using the technique of adoptive cell transfer, Loveland and colleagues (1981) have demonstrated that specifically sensitized T cells treated with anti- θ serum and complement were unable to mediate the rejection of established skin allografts carried by adult thymectomized, lethally irradiated and bone marrow reconstituted (ATXBM) mice. Conversely, untreated T cells sensitized against donor-specific alloantigens induced acute graft destruction. In our laboratory, a similar procedure has demonstrated that the rejection of established, cultured allografts of adult mouse islets is also T cell-dependent (Prowse *et al.*, 1982).

Whereas Lyt-1^+2^- T cells were shown to be the mediators for skin graft rejection (Loveland *et al.*, 1981), Lyt-1^+2^+ T cells are responsible for the rejection of cultured adult islets (Prowse *et al.*, 1982). In the latter situation, activation of any naive cells present in the inoculum is precluded because the cultured islet allograft is immunologically "silent". However, in the case of the skin graft experiments, the adoptive transfer of naive Lyt-1^+2^- T cells also results in graft rejection (Loveland and McKenzie, 1982a, 1982b). This finding indicates that activation of adoptively transferred T cells can occur in immunoincompetent ATXBM hosts bearing a skin allograft. As a consequence, the nature of the subclass of T cells mediating skin graft destruction in the ATXBM experimental system remains speculative. This limitation may also account for the anomaly that the adoptive transfer of sensitized Lyt-1^+2^- T cells to ATXBM mice induced the rejection of allogeneic EL-4 lymphoma (Loveland and McKenzie, 1982c), which does not express class II alloantigens (Section 1.3, Section 1.5.3).

While a role for sensitized Lyt-1^+2^+ T cells in the rejection of established allografts of cultured adult islets is conclusive, the nature of this rejection mechanism remains speculative. Graft damage may result from non-specific inflammatory responses due to lymphokine(s) released following recognition of graft alloantigen, or from direct cytotoxic activity (Section 1.6.1). Nash and Bell (1979) have reported indirect evidence for a role for macrophages in the rejection of allografts of adult rat islets. Indeed, graft destruction may, at least in part, be due to the participation of macrophages in the non-specific inflammatory response. Furthermore,

the rejection of established allografts of rat islets following the administration of third party, naive T cells (Zitron *et al.*, 1981b) may be due to non-specific damage following interaction with host blood cells perfusing the graft tissue. Similar interactions have been shown to damage cultured thyroid tissue but in these cases the inflammatory "crisis" subsided and the grafts recovered histological and functional integrity (Lafferty and Woolnough, 1977; Gose and Bach, 1981).

There is now good evidence from *in vitro* studies that two signals, alloantigen and a source of costimulator, are required to trigger lymphokine release from both Lyt-1^+2^- and Lyt-1^+2^+ T cell subsets (Lafferty *et al.*, 1980). Once again, this demonstration argues against Bach's postulate that Lyt-1^+2^- T cells require only one signal, the recognition of class II alloantigen, for activation (Section 1.5.3). Once activated, T cells belonging to either subset require only signal 1, recognition of alloantigen, to release lymphokine(s) (Lafferty *et al.*, 1980). Since graft rejection is a T cell-dependent process, it is evident that two signals are also required for activation of the rejection process, irrespective of whether the mechanism is lymphokine-mediated or involves direct cytotoxicity (Section 1.5.3; Section 1.6.1). As discussed earlier (Section 1.6.1), passenger leukocytes, present as either mobile or fixed lymphoreticular elements, represent the only cells of graft origin that have the capacity to deliver both signals required for T cell activation. Thus, our postulate that tissue immunogenicity is due to the presence of passenger blood cells is consistent with the *in vitro* requirements for the activation of both

lymphokine-producing and cytotoxic T cells.

Using the sponge matrix allograft model, Hopt and co-workers (1980) have demonstrated that activated T cells express a non-specific recruiting activity for both unsensitized and sensitized T cells. Furthermore, demonstration of this recruitment activity appeared to be dependent on an interaction between graft alloantigen and sensitized T cells of graft specificity (Hopt *et al.*, 1980; Hanto *et al.*, 1982). Such interactions also resulted in the release of factors, probably lymphokines, which influenced the permeability of the graft's vasculature, the regional blood flow and lymphocyte recruitment (Hopt *et al.*, 1981). These findings support the notion that the inflammatory component of the graft rejection process may be lymphokine-mediated (Section 1.4.2). Nevertheless, Ascher *et al.* (1981) have demonstrated that a cytotoxic effector cell response can develop within sponge matrix allografts. Consequently, it is possible that both the local release of lymphokine and cytotoxic activity contribute to graft destruction.

Established allografts of cultured mouse islets are not susceptible to antibody-mediated attack following the passive transfer of donor-specific antiserum in the presence or absence of an exogenous source of complement. Since donor endothelium degenerates during organ culture (Parr *et al.*, 1980b), this resistance has been attributed to the revascularization of the graft by host endothelium (Agostino *et al.*, 1982). Jooste *et al.* (1981a, 1981b) have shown conclusively that the hyperacute rejection of rat skin xenografts following the passive transfer of donor-specific

antiserum is due to an interaction between antibody and donor endothelium. The early period of resistance, from four to ten days after transplantation, was found to be due to immature properties of regenerating donor vascular endothelium (Jooste *et al.*, 1981a; Section 1.4.3). Using immunofluorescence techniques, they showed that the resistance of long-established grafts was attributable to the replacement of donor endothelium by host endothelial cells (Jooste *et al.*, 1981b). During the intervening period of susceptibility, it is likely that the formation of immune complexes between antibody and donor alloantigens expressed on the graft endothelium results in irreparable vascular injury (Section 1.4.3). Bogman *et al.* (1981) have proposed that the amount of anti-donor antibody present determines whether thrombosis, leading to vascular damage and ultimately graft failure, proceeds with or without the involvement of polymorphonuclear cells.

It is also evident that animals bearing long-term tissue allografts can become resistant to donor-specific immune stimulation. Bowen *et al.* (1981) demonstrated that some long-standing allografts of isolated adult mouse islets were not susceptible to rejection following either treatment of hosts with viable donor-specific peritoneal cells or non-specific immune stimulation of hosts by complete Freund's adjuvant. This demonstration supported Batchelor's finding that enhanced kidney allografts established for prolonged periods in secondary naive recipients failed to be rejected following challenge of the hosts with viable spleen cells, semi-isogeneic to the donor kidney (Batchelor *et al.*, 1979). Both studies have suggested that long-term exposure to alloantigen presented in a nonimmunogenic form i.e. on parenchymal graft cells, may

induce donor-specific unresponsiveness (Batchelor *et al.*, 1979; Bowen *et al.*, 1981). Further support for this notion has been provided by the demonstration that preimmunization of mice with donor-specific blood depleted of class II alloantigen-bearing cells indefinitely prolonged the survival of allografts of isolated adult islets, transplanted across a major histocompatibility barrier (Faustman *et al.*, 1982a). Similarly, in our laboratory, stabilization of cultured allografts of adult mouse islets has been reinforced by the administration of UV-treated donor-specific spleen cells (M. Agostino, personal communication). Together, these studies have demonstrated the induction of donor-specific unresponsiveness without the need for host immunosuppression.

The mechanism responsible for this nonresponsive state has been the subject of intense investigation. In our laboratory, the phenomenon has been studied in mice bearing long-term cultured thyroid allografts. Using this system, it has been shown that the nonresponsiveness is not due to antigenic modulation of the graft or to the removal of antigen-reactive cells (Donohoe *et al.*, 1982). Lacy's group has reported that spleen cells from hosts carrying long-term allografts of isolated adult rat islets can suppress donor-specific MLC reactivity of naive, isogeneic host lymphocytes (Zitron *et al.*, 1981a). Furthermore, they have attributed the rejection of established allografts of adult mouse islets following treatment of recipients with recipient-specific anti-*I-J* serum and donor-specific spleen cells to the removal of host suppressor cells (Faustman *et al.*, 1982b). While their data suggest a role for suppressor cells in maintaining the nonresponsive state of recipients bearing long-term islet

allografts, the present evidence is not conclusive. The characterization of these cells and investigation of their capacity to suppress donor-specific alloreactivity following adoptive transfer to naive recipients are steps which will lead to further clarification of this issue. Using such a procedure, Marquet and Heystek (1981) have demonstrated that the prolonged survival of rat heart allografts following pretreatment of recipients with donor-type whole blood can be attributed to a population of host suppressor T cells. Indeed, Dorsch and Roser (1982a, 1982b) have obtained indirect evidence that neonatally-induced tolerance to rat skin allografts is probably mediated by suppressor T cells derived from the tolerizing inoculum. Furthermore, they have suggested that this form of transplantation tolerance is due to anti-idiotypic responses directed against host receptors for alloantigens of donor-specificity (Dorsch and Roser, 1982b). Further studies in our laboratory on the nonresponsive state of mice carrying long-standing cultured islet allografts have indicated that these hosts are not fully tolerant; while secondary transplants of uncultured thyroid showed indefinite survival, allografts of 10 day-cultured foetal mouse pancreas were acutely rejected (M. Agostino, personal communication). We suggest that the rejection of only the cultured foetal pancreas is due to the presence of residual donor lymphoid elements which can elicit non-specific graft destruction (Section 1.6.1; Section 4.3). The allografts of adult islet tissue which constitute the "tolerizing inoculum" carry only class I donor alloantigens (Faustman *et al.*, 1980; Parr *et al.*, 1982). Consequently, nonresponsiveness to donor-type

class II alloantigens, and hence to foetal pancreas tissue, will not be induced. Whether suppressor T cells are involved in maintaining the nonresponsive state to cultured adult islet tissue in this transplant system is not known.

Batchelor and colleagues (1979) have demonstrated that the passive enhancement of rat renal allografts, following the administration of donor-specific antiserum around the time of transplantation, is due to inhibition at the level of stimulation and not to lack of target recognition. Hutchinson and Zola (1977) have formulated a mechanism of enhancement directed against antigen-reactive effector cells. They reported evidence which favours the sequestration and opsonization of antigen-reactive T cells in the liver. In accordance with these findings, it has been proposed that following binding of antigen-reactive cells to free graft antigen-antibody complexes, the former are opsonized by macrophages bound to the Fc portion of the same immune complexes (Hutchinson and Zola, 1977). Both these ideas have been assimilated into an alternative model of enhancement which postulates the opsonization of donor passenger leukocytes and interstitial dendritic cells (Guttmann and Lindquist, 1971; Hart and Fabre, 1982). In this manner, donor S^+ cells (Section 1.6.1) would be depleted and the graft would be rendered nonimmunogenic for T cell-dependent immune processes. From a logistical viewpoint, the extravascular localization of the dendritic cells may render this mechanism unfeasible. Interestingly, the location of the opsonizing process(es) in the liver may account for the induction of donor-specific nonresponsiveness following transplantation of orthotopic liver allografts in rats (Kamada *et al.*, 1981). The detection of donor-specific

antibodies in the serum of these transplant recipients is consistent with this notion (Kamada *et al.*, 1980).

Recently, the concept that passenger leukocytes represent the immunogenic component of transplant tissue has been challenged by the proposal that graft vascular endothelial cells may be responsible for stimulating allograft rejection (Hirschberg and Thorsby, 1981). Based on this notion, the reduction in tissue immunogenicity following organ culture has been attributed to the degeneration of vascular endothelium (Hirschberg and Thorsby, 1981; Rabinovitch *et al.*, 1982). However, the demonstration that long-term surviving kidney allografts retain donor-type vascular endothelium argues against this notion (Hart *et al.*, 1980; Section 1.6.3). The idea that vascular endothelial cells express S^+ activity was founded on the *in vitro* proliferative response of sensitized T cells to allogeneic endothelial cells (Hirschberg *et al.*, 1980). However, the point has already been made that sensitized T cells require only one signal, alloantigen recognition, for lymphokine release; furthermore, these cells proliferate in response to the lymphokine, interleukin 2 (IL2) (Andrus and Lafferty, 1981). It would appear that confusion over the "stimulatory" capacity of endothelial cells has stemmed from the misconception that class II alloantigens which are expressed on some endothelial tissues (Hirschberg *et al.*, 1980), including the capillaries found in islets (Alejandro *et al.*, 1982; Rabinovitch *et al.*, 1982), constitute the second signal required for T cell activation (Alejandro *et al.*, 1982; Rabinovitch *et al.*, 1982). Indeed, we have already presented considerable evidence which fails to uphold this postulate of

Bach's model for the pathway of T cell activation (Section 1.5.3).

In summary, the immunogenicity of tissue allografts can be attributed to the presence of donor passenger leukocytes. Accordingly, our studies indicate that tissue-specific differences in immunogenicity are related to the degree of leukocyte contamination. We have demonstrated that modulation of the immunogenicity of both foetal and adult pancreatic islet tissue can be achieved by treating the tissue, prior to transplantation, to remove lymphoreticular elements. In terms of the possible clinical application of pancreatic islet transplantation for the treatment of diabetes mellitus, there is now good experimental evidence that the need for host immunosuppression (Section 1.7) may be dismissed or at least minimized by utilizing procedures which reduce the immunogenicity of the islet tissue before transplantation. In addition, experimental studies in rodents have demonstrated that transplantation of both foetal (Mandel *et al.*, 1981) and adult (Gray and Watkins, 1976b) islet tissue to diabetic hosts can protect them from the development of secondary, vascular complications of diabetes. At present, the foetal pancreas appears to be the more suitable source of islet tissue for clinical transplantation (Section 1.7). Our demonstration that foetal proislets, unlike foetal pancreas, are weakly immunogenic leads us to propose that the isolation of proislets represents a promising approach for the allotransplantation of foetal islet tissue. Ultimately, the feasibility of pancreatic islet transplantation for the treatment of diabetes mellitus will rest with the elucidation of the etiology and pathogenesis of the disease.

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- ADELMAN, N.E., HAMMOND, M.E., COHEN, S. and DVORAK, H.F.
(1979) Lymphokines as inflammatory mediators. In:
Biology of the lymphokines. Cohen, S., Pick, E. and
Oppenheim, J.J. (Eds). Academic Press, New York,
pp. 13-58.
- AGOSTINO, M., PROWSE, S.J. and LAFFERTY, K.J. (1982)
Resistance of established islet allografts to rejection
by antibody and complement. Aust. J. Exp. Biol. Med.
Sci. 60 : 219-222.
- ALEJANDRO, R., SCHIENVOLD, F.L., HAJEK, S.V., RYAN, U.,
MILLER, J. and MINTZ, D.H. (1982) Immunocytochemical
localization of HLA-DR in human islets of Langerhans.
Diabetes 31 (Suppl. 4) : 17-22.
- ANDERSSON, A. (1979) Survival of pancreatic islet allografts.
Lancet 1 : 585.
- ANDERSSON, A. and BUSCHARD, K. (1977) Culture of isolated
pancreatic islets: Its applications for transplantation
purposes. Trans. Am. Soc. Artif. Intern. Organs 23 :
342-346.
- ANDERSSON, A. and HELLERSTRÖM, C. (1980) Explant culture:
Pancreatic islets. Methods in cell biology 21B :
135-151.
- ANDRUS, L. and LAFFERTY, K.J. (1981) Interleukin 2 production
by alloantigen (H-2) activated T cells. Aust. J. Exp.
Biol. Med. Sci. 59 : 413-426.
- ASCHER, N.L., FERGUSON, R.M., HOFFMAN, R. and SIMMONS, R.L.
(1979) Partial characterisation of cytotoxic cells
infiltrating sponge matrix allografts. Transplantation
27 : 254-259.
- ASCHER, N.L., CHEN, S., HOFFMAN, R. and SIMMONS, R.L. (1981)
Maturation of cytotoxic effector cells at the site of
allograft rejection. Transplant. Proc. 13 : 1105-1107.
- BACH, F. and HIRSCHHORN (1964) Lymphocyte interaction: A
potential histocompatibility test *in vitro*. Science
143 : 813-814.

- BACH, F.H. and ALTER, B.J. (1978) Alternative pathways of T lymphocyte activation. *J. Exp. Med.* 148 : 829-834.
- BACH, F.H., BACH, M.L. and SONDEL, P.M. (1976) Differential function of major histocompatibility complex antigens in T-lymphocyte activation. *Nature* 259 : 273-281.
- BACH, F.H., ALTER, B.J. and SOPORI, M.L. (1979) T lymphocyte subpopulations responsive to alloantigens. *J. Reticuloendothelial Soc.* 26 : 587-596.
- BAIN, B., VAS, M.R. and LOWENSTEIN, L. (1964) The development of large immature mononuclear cells in mixed leukocyte cultures. *Blood* 23 : 108-116.
- BARKER, C.F. and BILLINGHAM, R.E. (1970) Comparison of the fates of Ag-B locus compatible homografts of skin and hearts in inbred rats. *Nature* 225 : 851-852.
- BARKER, C.F. and BILLINGHAM, R.E. (1971) Histocompatibility requirements of heart and skin grafts in rats. *Transplant. Proc.* 3 : 172-175.
- BATCHELOR, J.R. (1978) The riddle of kidney graft enhancement. *Transplantation* 26 : 139-141.
- BATCHELOR, J.R., WELSH, K.I. and BURGOS, H. (1978) Transplantation antigens *per se* are poor immunogens within a species. *Nature* 273 : 54-56.
- BATCHELOR, J.R., WELSH, K.I., MAYNARD, A. and BURGOS, H. (1979) Failure of long-surviving, passively enhanced kidney allografts to provoke T-dependent alloimmunity. I. Retransplantation of (AS x AUG) F₁ kidneys into secondary AS recipients. *J. Exp. Med.* 150 : 455-464.
- BEVAN, M.J. (1975) The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J. Exp. Med.* 142 : 1349-1364.
- BEVAN, M.J. (1976a) Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* 143 : 1283-1288.

- BEVAN, M.J. (1976b) Minor H antigens introduced on H-2 different stimulating cells cross-react at the cytotoxic T cell level during *in vivo* priming. J. Immunol. 117 : 2233-2238.
- BILLINGHAM, R.E. (1961) Free skin grafting in mammals (Chapter 1). In: Transplantation of tissues and cells. Billingham, R.E. and Silvers, W.K. (Eds). The Wistar Institute Press, Philadelphia, pp. 1-26.
- BILLINGHAM, R.E. (1971) The passenger cell concept in transplantation immunology. Cell. Immunol. 2 : 1-12.
- BILLINGHAM, R.E. (1976) The feasibility of altering the immunogenicity of grafts. J. Invest. Dermatol. 67 : 149-159.
- BILLINGHAM, R.E. and PARKES, A.S. (1955) Studies on the survival of homografts of skin and ovarian tissue in rats. Proc. Roy. Soc. B. 143 : 550-560.
- BILLINGHAM, R.E. and SILVERS, W.K. (1964) Studies on homografts of foetal and infant skin and further observations on the anomalous properties of pouch skin grafts in hamsters. Proc. Roy. Soc. B. 161 : 168-190.
- BILLINGHAM, R.E., BRENT, L. and MEDAWAR, P.B. (1953) 'Actively acquired tolerance' of foreign cells. Nature 172 : 603-606.
- BILLINGHAM, R.E., BRENT, L. and MEDAWAR, P.B. (1954) Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. Proc. Roy. Soc. B. 143 : 58-80.
- BILLINGHAM, R.E., BRENT, L. and MEDAWAR, P.B. (1956a) Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. Phil. Trans. Roy. Soc. B. 239 : 357-414.

- BILLINGHAM, R.E., BRENT, L. and MEDAWAR, P.B. (1956b)
'Enhancement' in normal homografts, with a note on its possible mechanism. *Transplantation Bull.* 3 : 84-88.
- BILLINGHAM, R.E., BRENT, L. and MEDAWAR, P.B. (1958)
Extraction of antigens causing transplantation immunity. *Transplantation Bull.* 5 : 377-381.
- BOGMAN, M.J.J.T., BERDEN, J.H.M., HAGEMANN, J.F.H.M.,
MAASS, C.N. and KOENE, R.A.P. (1980) Patterns of vascular damage in the antibody-mediated rejection of skin xenografts in the mouse. *Am. J. Pathol.* 100 : 727-738.
- BOWEN, K.M. and LAFFERTY, K.J. (1980) Reversal of diabetes by allogeneic islet transplantation without immunosuppression. *Aust. J. Exp. Biol. Med. Sci.* 58 : 441-447.
- BOWEN, K.M., ANDRUS, L. and LAFFERTY, K.J. (1980) Successful allotransplantation of mouse pancreatic islets to nonimmunosuppressed recipients. *Diabetes* 29 (Suppl. 1): 98-104.
- BOWEN, K.M., PROWSE, S.J. and LAFFERTY, K.J. (1981) Reversal of diabetes by islet transplantation: Vulnerability of the established allograft. *Science* 213 : 1261-1262.
- BOYSE, E.A., LANCE, E.M., CARSWELL, E.A., COOPER, S. and OLD, L.J. (1970) Rejection of skin allografts by radiation chimaeras: Selective gene action in the specification of cell surface structure. *Nature* 227 : 901-903.
- BRACIALE, T.J. and YAP, K.L. (1978) Role of viral infectivity in the induction of influenza virus-specific cytotoxic T cells. *J. Exp. Med.* 147 : 1236-1252.
- BRETSCHER, P. and COHN, M. (1970) A theory of self-nonsel self discrimination. *Science* 169 : 1042-1049.
- BROE, P.J., MEHIGAN, D.G. and CAMERON, J.L. (1981) Pancreatic transplantation. *Surg. Clin. North Amer.* 61 : 85-98.
- BROWN, J., MOLNAR, I.G., CLARK, W. and MULLEN, Y. (1974) Control of experimental diabetes mellitus in rats by transplantation of fetal pancreases. *Science* 184 : 1377-1379.

- BROWN, J., CLARK, W.R., MOLNAR, I.G. and MULLEN, Y.S. (1976) Fetal pancreas transplantation for reversal of Streptozotocin-induced diabetes in rats. *Diabetes* 25 : 56-64.
- BROWN, J., HEININGER, D., KURET, J. and MULLEN, Y. (1981) Islet cells grow after transplantation of fetal pancreas and control of diabetes. *Diabetes* 30 : 9-13.
- BROWNING, H. and RESNIK, P. (1951-1952) Homologous and heterologous transplantation of pancreatic tissue in normal and diabetic mice. *Yale J. Biol. Med.* 24 : 141-152.
- BUNNAG, S.C. (1966) Postnatal neogenesis of islets of Langerhans in the mouse. *Diabetes* 15 : 480-491.
- BURDICK, J.F., RUSSELL, P.S. and WINN, H.J. (1979) Sensitivity of long-standing xenografts of rat hearts to humoral antibodies. *J. Immunol.* 123 : 1732-1735.
- BURNET, F.M. and FENNER, F. (1949) Theoretical aspects of antibody production (Chapter 8). In: *The production of antibodies*. Macmillan, Melbourne, pp. 78-106.
- BURNET, F.M., STONE, J.D. and EDNEY, M. (1950) The failure of antibody production in the chick embryo. *Aust. J. Exp. Biol. Med. Sci.* 28 : 291-297.
- BUSSOLATI, G. and BASSA, T. (1974) Thiosulfation aldehyde fuchsin (TAF) procedure for the staining of pancreatic B cells. *Stain Tech.* 49 : 313-315.
- CALNE, R.Y., WHITE, H.J.O., BINNS, R.M., HERBERTSON, B.M., MILLARD, P.R., PENA, J., SALAMAN, J.R., SAMUEL, J.R. and DAVIS, D.R. (1969) Immunosuppressive effects of the orthotopically transplanted porcine liver. *Transplant. Proc.* 1 : 321-324.
- CANTOR, H. and BOYSE, E.A. (1975) Functional subclasses of T lymphocytes bearing different Ly antigens. 1. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* 141 : 1376-1389.

- CARLSON, G.A. and TERRES, G. (1976) Antibody-induced killing *in vivo* of L1210/MTX-R cells quantitated in passively immunized mice with ^{131}I -iododeoxyuridine-labelled cells and whole-body measurement of retained radioactivity. *J. Immunol.* 117 : 822-829.
- CARPENTER, C.B. (1981) The cellular basis of allograft rejection. *Immunology Today* 2 : 50-54.
- CARPENTER, C.B., d'APICE, A.J.F. and ABBAS, A.K. (1976) The role of antibodies in the rejection and enhancement of organ allografts. *Adv. Immunol.* 22 : 1-65.
- CEROTTINI, J-C., NORDIN, A.A. and BRUNNER, K.T. (1970) Specific *in vitro* cytotoxicity of thymus-derived lymphocytes sensitised to alloantigens. *Nature* 228 : 1308-1309.
- CHASE, H.P., OCRANT, I. and TALMAGE, D.W. (1979) The effects of different conditions of organ culture on the survival of the mouse pancreas. *Diabetes* 28 : 990-993.
- CONNOLLY, J.E., MARTIN, D.C., STEINBERG, T., GWINUP, G., GAZZANIGA, A.B. and BARTLETT, R.H. (1973) Clinical experience with pancreaticoduodenal transplantation. *Arch. Surg.* 106 : 489-494.
- CORLEY, R.B., DAWSON, J.R. and AMOS, D.B. (1975) Lymphocyte stimulation *in vitro*: Generation of cytotoxic effector lymphocytes using subcellular fractions of a lymphoid cell line. *Cell. Immunol.* 16 : 92-105.
- COUPLAND, R.E. (1960) The survival and growth of pancreatic tissue in the anterior chamber of the eye of the albino rat. *J. Endocrinology* 20 : 69-77.
- DANILOVS, J.A., HOFMAN, F.M., TAYLOR, C.R. and BROWN, J. (1982) Expression of HLA-DR antigens in human fetal pancreas tissue. *Diabetes* 31 (Suppl. 4) : 23-28.

- DAY, E.D., KALISS, N., ARONSON, A.I., BRYANT, B.F., FRIENDLY, D., GABRIELSON, F.C. and SMITH, P.M. (1954) Investigations of substances in mouse tissues inducing alteration of normal host-homograft relationships. J. Nat. Cancer Inst. 15 : 145-159.
- DONOHUE, J.A., ANDRUS, L., BOWEN, K.M., SIMEONOVIC, C.J., PROWSE, S.J. and LAFFERTY, K.J. (1982) Cultured thyroid allografts induce a state of partial tolerance in adult recipient mice. Transplantation. In press.
- DORSCH, S. and ROSER, B. (1982a) Suppressor cells in transplantation tolerance. I. Analysis of the suppressor status of neonatally and adoptively tolerized rats. Transplantation 33 : 518-524.
- DORSCH, S. and ROSER, B. (1982b) Suppressor cells in transplantation tolerance. II. Identification and probable mode of action of chimeric suppressor T cells. Transplantation 33 : 525-529.
- ELKINS, W.L. (1966) The interaction of donor and host lymphoid cells in the pathogenesis of renal cortical destruction induced by a local graft versus host reaction. J. Exp. Med. 123 : 103-118.
- ELKINS, W.L. (1971) The sources of immunogenic stimulation of lymphoid cells mediating a local graft-versus-host reaction in rat kidney. Transplantation 11 : 551-560.
- ELKINS, W.L. and GUTTMANN, R.D. (1968) Pathogenesis of a local graft versus host reaction: Immunogenicity of circulating host leukocytes. Science 159 ; 1250-1251.
- EMMA, D.A. and JACOBS, B.B. (1981) Prolongation of skin allograft survival following donor irradiation and organ culture explantation. Transplantation 31 : 138-139.
- ENGERS, H.D., THOMAS, K., CEROTTINI, J-C. and BRUNNER, K.T. (1975) Generation of cytotoxic T lymphocytes *in vitro*. V. Response of normal and immune spleen cells to subcellular alloantigens. J. Immunol. 115 : 356-360.

- FAST, L.D. and FAN, D.P. (1978) Dissociated and reconstituted subcellular alloantigen capable of stimulating mouse cytotoxic T lymphocytes *in vitro*. J. Immunol. 120 : 1092-1096.
- FAUSTMAN, D., HAUPTFELD, V., DAVIE, J.M., LACY, P.E. and SCHREFFLER, D.C. (1980) Murine pancreatic β -cells express H-2K and H-2D but not *Ia* antigens. J. Exp. Med. 151 : 1563-1568.
- FAUSTMAN, D., HAUPTFELD, V., LACY, P. and DAVIE, J. (1981) Prolongation of murine islet allograft survival by pretreatment of islets with antibody directed to *Ia* determinants. Proc. Natl. Acad. Sci. U.S.A. 78 : 5156-5159.
- FAUSTMAN, D., LACY, P., DAVIE, J. and HAUPTFELD, V. (1982a) Prevention of allograft rejection by preimmunization with donor blood depleted of *Ia*-bearing cells. Science 217 : 157-158.
- FAUSTMAN, D., HAUPTFELD, V., LACY, P. and DAVIE, J. (1982b) Demonstration of active tolerance in maintenance of established islet of Langerhans allografts. Proc. Natl. Acad. Sci. U.S.A. 79 : 4153-4155.
- FELDMAN, S.D., HIRSCHBERG, G.E., DODI, G., RAIZMAN, M.E., SCHARP, D., BALLINGER, W.F. and LACY, P.E. (1977) Intrasplenic islet isografts. Surgery 82 : 386-394.
- FERGUSON, J., ALLSOPP, R.H., TAYLOR, R.M.R. and JOHNSTON, I.D.A. (1977) Isolation of viable human pancreatic islets. World J. Surg. 1 : 69-77.
- FINKE, E.H., LACY, P.E. and ONO, J. (1979) Use of reflected green light for specific identification of islets *in vitro* after collagenase isolation. Diabetes 28 : 612-613.
- FRANGIPANE, L.G., BARKER, C.F. and SILVERS, W.K. (1977) Importance of weak histocompatibility factors in survival of pancreatic islet transplants. Surg. Forum 28 : 294-296.

- FREEMAN, J.S., CHAMBERLAIN, E.C., REEMTSMA, K. and STEINMULLER, D. (1971) Prolongation of rat heart allografts by donor pretreatment with immunosuppressive agents. *Transplant. Proc.* 3 : 580-582.
- FRENCH, M.E. (1972) The early effects of alloantibody and complement on rat kidney allografts. *Transplantation* 13 : 447-451.
- GAILLARD, P.J. (1948) Growth, differentiation and function of explants of some endocrine glands. *Sym. Soc. Exp. Biol.* 2 : 139-144.
- GARVEY, J.F.W., MORRIS, P.J. and MILLARD, P.R. (1979a) Early rejection of allogeneic foetal rat pancreas. *Transplantation* 27 : 342-344.
- GARVEY, J.F.W., MORRIS, P.J., FINCH, D.R.A., MILLARD, P.R. and POOLE, M. (1979b) Experimental pancreas transplantation. *Lancet* 1 : 971-972.
- GARVEY, J.F.W., MILLARD, P.R. and MORRIS, P.J. (1980a) Experimental transplantation of fetal pancreas and isolated islets in the rat: Studies of donor pretreatment and recipient immunosuppression. *Transplant. Proc.* 12 : 186-189.
- GARVEY, J.F.W., KLEIN, C., MILLARD, P.R. and MORRIS, P.J. (1980b) Rejection of organ-cultured allogeneic fetal rat pancreas. *Surgery* 87 : 157-163.
- GERLAG, P.G.G., KOENE, R.A.P., HAGEMANN, J.F.H.M. and WIJDEVELD, P.G.A.B. (1975) Hyperacute rejection of skin allografts in the mouse. *Transplantation* 20 : 308-313.
- GOLDMAN, H. and COLLE, E. (1976) Human pancreatic islets in culture: Effects of supplementing the medium with homologous and heterologous serum. *Science* 192 : 1014-1016.
- GOLDSMITH, K.L.G. (1965) Donor selection and compatibility typing. *Brit. Med. Bull.* 21 : 162-165.
- GORDON, R.D., MATHIESON, B.J., SAMELSON, L.E., BOYSE, E.A. and SIMPSON, E. (1976) The effect of allogeneic presensitisation on H-Y graft survival and *in vitro* cell-mediated responses to H-Y antigen. *J. Exp. Med.* 144 : 810-820.

- GORER, P.A. (1937) The genetic and antigenic basis of tumour transplantation. *J. Pathol. and Bacteriol.* 44 : 691-697.
- GORER, P.A., LYMAN, S. and SNELL, G.D. (1948) Studies on the genetic and antigenic basis of tumour transplantation. Linkage between a histocompatibility gene and "fused" in mice. *Proc. Roy. Soc. B.* 135 : 499-505.
- GOSE, J.E. and BACH, F.H. (1981) Rejection crises that resolve can be triggered by passenger cells in otherwise syngeneic transplants. *Transplant. Proc.* 13 : 1117-1119.
- GOWANS, J.L., MCGREGOR, D.D. and COWEN, D.M. (1962) Initiation of immune responses by small lymphocytes. *Nature* 196 : 651-655.
- GRAFF, R.J. (1970) Polymorphism of histocompatibility genes in the mouse. *Transplant. Proc.* 2 : 15-23.
- GRAY, B.N. and WATKINS, E., Jr. (1976a) Isolated islet transplantation in experimental diabetes. *Aust. J. Exp. Biol. Med. Sci.* 54 : 57-70.
- GRAY, B.N. and WATKINS, E. (1976b) Prevention of vascular complications of diabetes by pancreatic islet transplantation. *Arch. Surg.* 111 : 254-257.
- GREINER, D.K. and ROSENTHAL, A.S. (1975) Macrophage activation of allogeneic lymphocyte proliferation in the guinea pig mixed leukocyte culture. *J. Immunol.* 114 : 1541-1547.
- GROTH, C.G., ANDERSSON, A., BJÖRKEN, C., GUNNARSSON, R., HELLERSTROM, C., LUNDGREN, G., PETERSSON, B., SWENNE, I. and ÖSTMAN, J. (1980) Transplantation of fetal pancreatic microfragments via the portal vein to a diabetic patient. *Diabetes* 29 (Suppl. 1) : 80-83.
- GUTTMANN, R.D. and LINDQUIST, R.R. (1971) Hematopoietic origin of immunological target in enhancement of renal allografts. *Transplant. Proc.* 3 : 717-720.

- GUTTMANN, R.D., CARPENTER, C.B., LINDQUIST, R.R. and MERRILL, J.P. (1967) An immunosuppressive site of action of heterologous antilymphocyte serum. *Lancet* 1 : 248-249.
- GUTTMANN, R.D., BEAUDOIN, J.G., MOREHOUSE, D.D., KLASSEN, J., KNAACK, J., JEFFERY, J., CHASSOT, P.G. and ABBOU, C.C (1975) Donor pretreatment as an adjunct to cadaver renal allotransplantation. *Transplant. Proc.* 7 : 117-121.
- HALL, B.M., DORSCH, S. and ROSER, B. (1978a) The cellular basis of allograft rejection *in vivo*. I. The cellular requirements for first-set rejection of heart grafts. *J. Exp. Med.* 148 : 878-889.
- HALL, B.M., DORSCH, S. and ROSER, B. (1978b) The cellular basis of allograft rejection *in vivo*. II. The nature of memory cells mediating second set heart graft rejection. *J. Exp. Med.* 148 : 890-902.
- HÄMMERLING, G.J. (1976) Tissue distribution of *Ia* antigens and their expression on lymphocyte subpopulations. *Transplant. Rev.* 30 : 64-82.
- HANKS, J.H. and WALLACE, R.E. (1949) Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. Med.* 71 : 196-200.
- HANTO, D.W., HOPT, U.T., HOFFMAN, R. and SIMMONS, R.L. (1982) Recruitment of unsensitized circulating lymphocytes to sites of allogeneic cellular interactions. *Transplantation* 33 : 541-546.
- HARDY, D.A. and LING, N.R. (1969) Effects of some cellular antigens on lymphocytes and the nature of the mixed lymphocyte reaction. *Nature* 221 : 545-548.
- HARDY, D.A., KNIGHT, S. and LING, N.R. (1970) The interaction of normal lymphocytes and cells from lymphoid cell lines. *Immunology* 19 : 329-342.
- HART, D.N.J. and FABRE, J.W. (1981) Demonstration and characterization of *Ia*-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. *J. Exp. Med.* 153 : 347-361.

- HART, D.N.J. and FABRE, J.W. (1982) Mechanism of induction of passive enhancement. Evidence for an interaction of enhancing antibody with donor interstitial dendritic cells. *Transplantation* 33 : 319-322.
- HART, D.N.J., WINEARLS, C.G. and FABRE, J.W. (1980) Graft adaptation: Studies on possible mechanisms in long-term surviving rat renal allografts. *Transplantation* 30 : 73-80.
- HÁŠKOVÁ, V. and HINZOVÁ, E. (1966) Prolonged survival and tolerogenic action of skin grafts from newborn donors in adult mice. *Folia Biol.* 12 : 29-34.
- HATTLER, B.G., ROCKLIN, R.E., WARD, P.A. and RICKLES, F.R. (1973) Functional features of lymphocytes recovered from a human renal allograft. *Cell. Immunol.* 9 : 289-296.
- HÄYRY, P. and DEFENDI, V. (1970) Mixed lymphocyte cultures produce effector cells: Model *in vitro* for allograft rejection. *Science* 168 : 133-135.
- HÄYRY, P. and ANDERSSON, L.C. (1976) Generation of T memory cells in one-way mixed lymphocyte culture. IV. Primary and secondary responses to soluble and insoluble membrane preparations and to ultraviolet-light-inactivated stimulator cells. *Scand. J. Immunol.* 5 : 391-399.
- HÄYRY, P., ANDERSSON, L.C., NORDLING, S. and VIROLAINEN, M. (1972) Allograft response *in vitro*. *Transplant. Rev.* 12 : 91-140.
- HÄYRY, P., VON WILLEBRAND, E. and SOOTS, A. (1979) *In situ* effector mechanisms in rat kidney allograft rejection. III. Kinetics of the inflammatory response and generation of donor-directed killer cells. *Scand. J. Immunol.* 10 : 95-108.
- HEGRE, O.D., McEVOY, R.C., BACHELDER, V. and LAZAROW, A. (1972) Organ culture of fetal rat pancreas: Quantitative analysis by linear scanning of islet and other tissue components. *In Vitro* 7 : 366-376.

- HEGRE, O.D., LEONARD, R.J., ERLANDSEN, S.L., McEVOY, R.C., PARSONS, J.A., ELDE, R.P. and LAZAROW, A. (1976a) Transplantation of islet tissue in the rat. *Acta Endocrinol. Copenh. Suppl.* 205 : 257-281.
- HEGRE, O.D., LEONARD, R.J., RUSIN, J.D. and LAZAROW, A. (1976b) Transplantation of the fetal rat pancreas: Quantitative morphological analysis of islet tissue growth. *Anat. Rec.* 185 : 209-222.
- HEGRE, O.D., SCHMITT, R.V. and McEVOY, R.C. (1979) Syngeneic transplantation of the fetal rat pancreas. IV. Dissociated versus whole organ implantation. *Metabolism* 28 : 157-162.
- HEGRE, O.D., SCHULTE, B.A., PARSONS, U.A. and ERLANDSEN, S.L. (1981) The preservation and isolation of perinatal islet tissue utilizing *in vitro* techniques. In: Islet isolation, culture and cryopreservation. International Workshop Giessen, 1980. Federlin, K. and Bretzel, R.G. (Eds). Thieme, Stuttgart, pp. 83-95.
- HEINZE, E. and STEINKE, J. (1971) Glucose metabolism of isolated pancreatic islets: Difference between fetal, newborn and adult rats. *Endocrinology* 88 : 1259-1263.
- HELLERSTRÖM, C., LEWIS, N.J., BORG, H., JOHNSON, R. and FREINKEL, N. (1979) Method for large scale isolation of pancreatic islets by tissue culture of fetal rat pancreas. *Diabetes* 28 : 769-776.
- HESLOP, B.F. (1969) Maturation of histocompatibility antigens and host reactivity in the rat. *Transplant. Proc.* 1 : 560-563.
- HIRSCHBERG, H. and THORSBY, E. (1981) Immunogenicity of foreign tissues. *Transplantation* 31 : 96-97.
- HIRSCHBERG, H., BERGH, O.J. and THORSBY, E. (1980) Antigen-presenting properties of human vascular endothelial cells. *J. Exp. Med.* 152 : 249s-255s.

- HOFFMAN, R., ASCHER, N. and SIMMONS, R.L. (1979) *In vitro* chemotaxis of lymphocytes to fluid factors in rejecting allografts. In: The molecular basis of immune cell function. Proc. of the 13th International Leukocyte Culture Conference, 1979. Kaplan, J.G. (Ed.). Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 545-548.
- HOFFMAN, L., MARTIN, F.I.R., CARTER, W., MANDEL, T.E. and CAMPBELL, D.G. (1981) Effect of short-term insulin treatment on the reversal of diabetes after transplantation of syngeneic cultured or uncultured fetal mouse pancreas. *Transplantation* 32 : 342-345.
- HOPT, U.T., SULLIVAN, W., HOFFMAN, R. and SIMMONS, R.L. (1980) Migration and cell recruiting activity of specifically sensitized lymphocytes in sponge matrix allografts. *Transplantation* 30 : 411-416.
- HOPT, U.T., BOCKHORN, H. and SIMMONS, R.L. (1981) Effect of specifically sensitized lymphocytes on vascular permeability, regional blood flow and lymphocyte recruitment in sponge matrix allografts. *Immunobiology* 159 : 147.
- HUTCHINSON, I.V. and ZOLA, H. (1977) Antigen-reactive cell opsonization (ARCO). A mechanism of immunological enhancement. *Transplantation* 23 : 464-469.
- ISAKOV, N., YANKELEVICH, B., SEGAL, S. and FELDMAN, M. (1979) Differential immunogenic expression of an H-2-linked histocompatibility antigen on different tissues. *Transplantation* 28 : 31-35.
- JACOBS, B.B. (1974) Ovarian allograft survival. *Transplantation* 18 : 454-457.
- JACOBS, B.B. and HUSEBY, R.A. (1967) Growth of tumors in allogeneic hosts following organ culture explantation. *Transplantation* 5 : 410-419.
- JACOBS, B.B. and HUSEBY, R.A. (1968) Successful growth of tumour allografts following explantation: Effect of various culture conditions. *Proc. Soc. Exp. Biol. Med.* 127 : 957-960.

- JONASSON, O. (1979) Transplantation of the pancreas. Transplant. Proc. 11 : 325-330.
- JONASSON, O. and HOVERSTEN, G.H. (1978) Replacement of pancreatic beta cells as treatment for diabetes mellitus. A review. Surg. Ann. 10 : 1-21.
- JOOSTE, S.V. and WINN, H.J. (1975) Acute destruction of rat skin grafts by alloantisera. J. Immunol. 114 : 933-938.
- JOOSTE, S.V., COLVIN, R.B., SOPER, W.D. and WINN, H.J. (1981a) The vascular bed as the primary target in the destruction of skin grafts by antiserum. I. Resistance of freshly placed xenografts of skin to antiserum. J. Exp. Med. 154 : 1319-1331.
- JOOSTE, S.V., COLVIN, R.B. and WINN, H.J. (1981b) The vascular bed as the primary target in the destruction of skin grafts by antiserum. II. Loss of sensitivity to antiserum in long-term xenografts of skin. J. Exp. Med. 154 : 1332-1341.
- KALISS, N. and KANDUTSCH, A.A. (1956) Acceptance of tumour homografts by mice injected with antiserum. I. Activity of serum fractions. Proc. Soc. Exp. Biol. Med. 91 : 118-121.
- KAMADA, N., BRONS, G. and DAVIES, H.ff.S. (1980) Fully allogeneic liver grafting in rats induces a state of systemic nonreactivity to donor transplantation antigens. Transplantation 29 : 429-431.
- KAMADA, N., DAVIES, H.ff.S. and ROSER, B. (1981) Reversal of transplantation immunity by liver grafting. Nature 292 : 840-842.
- KASAKURA, S. and LOWENSTEIN (1968) The factors affecting the strength of "one-way" stimulation with irradiated leukocytes in mixed leukocyte cultures. J. Immunol. 101 : 12-17.
- KEDINGER, M., HAFFEN, K., GRENIER, J. and ELOY, R. (1977) *In vitro* culture reduces immunogenicity of pancreatic endocrine islets. Nature 270 : 736-738.

- KENDALL, M.G. and STUART, A. (1973) Categorical data (Chapter 33). In: The advanced theory of Statistics. Volume 2; Inference and relationship. Charles Griffin Co. Ltd, London, pp 556-611.
- KISSMEYER-NIELSEN, F., OLSEN, S., PETERSEN, V.P. and FJELDBORG, O. (1966) Hyperacute destruction of kidney allografts, associated with pre-existing humoral antibodies against donor cells. *Lancet* 2 : 662-665.
- KLEIN, J. (1972) Is the H-2K locus of the mouse stronger than the H-2D locus? *Tissue Antigens* 2 : 262-266.
- KLEIN, J. (1975a) Histocompatibility system of the mouse (Chapter 8). In: Biology of the mouse histocompatibility-2 complex. Springer-Verlag, New York, pp. 151-177.
- KLEIN, J. (1975b) Terra incognita: Function and evolution of the H-2 complex (Chapter 20). In: Biology of the the mouse histocompatibility-2 complex. Springer-Verlag, New York, pp. 521-536.
- KLEIN, J. (1979) The major histocompatibility complex of the mouse. *Science* 203 : 516-521.
- KLEIN, J., CHIANG, C., LOFGREEN, J. and STEINMULLER, D. (1976) Participation of H-2 regions in heart-transplant rejection. *Transplantation* 22 : 384-390.
- KLEIN, J., JURETIĆ, A., BAXEVANIS, C.N. and NAGY, Z.A. (1981) The traditional and a new version of the mouse H-2 complex. *Nature* 291 : 455-460.
- KYGER, E.R. and SALYER, K.E. (1973) The role of donor passenger leukocytes in rat skin allograft rejection. *Transplantation* 16 : 537-543.
- LACY, P.E., DAVIE, J.M. and FINKE, E.H. (1979a) Prolongation of islet allograft survival following *in vitro* culture (24°C) and a single injection of ALS. *Science* 204 : 312-313.

- LACY, P.E., DAVIE, J.M., FINKE, E.H. and SCHARP, D.W. (1979b) Prolongation of islet allograft survival. *Transplantation* 27 : 171-174.
- LACY, P.E., DAVIE, J.M. and FINKE, E.H. (1979c) Induction of rejection of successful allografts of rat islets by donor peritoneal exudate cells. *Transplantation* 28 : 415-420.
- LACY, P.E., FINKE, E.H., JANNEY, C.G. and DAVIE, J.M. (1982) Prolongation of islet xenograft survival by *in vitro* culture of rat megaislets in 95% O₂. *Transplantation* 33 : 588-592.
- LAFFERTY, K.J. and JONES, M.A.S. (1969) Reactions of the graft versus host (GVH) type. *Aust. J. Exp. Biol. Med. Sci.* 47 : 17-54.
- LAFFERTY, K.J. and CUNNINGHAM, A.J. (1975) A new analysis of allogeneic interactions. *Aust. J. Exp. Biol. Med. Sci.* 53 : 27-42.
- LAFFERTY, K.J. and TALMAGE, D.W. (1976) Theory of allogeneic reactivity and its relevance to transplantation biology. *Transplant. Proc.* 3 : 349-353.
- LAFFERTY, K.J. and WOOLNOUGH, J. (1977) The origin and mechanism of the allograft reaction. *Immunological Rev.* 35 : 231-262.
- LAFFERTY, K.J., WALKER, K.Z., SCOLLAY, R.G. and KILLBY, V.A.A. (1972) Allogeneic interactions provide evidence for a novel class of immunological reactivity. *Transplant. Rev.* 12 : 198-228.
- LAFFERTY, K.J., MISKO, I.S. and COOLEY, M.A. (1974) Allogeneic stimulation modulates the *in vitro* response of T cells to transplantation antigen. *Nature* 249 : 275-276.
- LAFFERTY, K.J., COOLEY, M.A., WOOLNOUGH, J. and WALKER, K.Z. (1975) Thyroid allograft immunogenicity is reduced after a period in organ culture. *Science* 188 : 259-261.

- LAFFERTY, K.J., BOOTES, A., KILLBY, V.A.A. and BURCH, W.
(1976a) Mechanism of thyroid allograft rejection.
Aust. J. Exp. Biol. Med. Sci. 54 : 573-586.
- LAFFERTY, K.J., BOOTES, A., DART, G. and TALMAGE, D.W.
(1976b) Effect of organ culture on the survival of
thyroid allografts in mice. Transplantation 22 :
138-149.
- LAFFERTY, K.J., WARREN, H.S., WOOLNOUGH, J.A. and TALMAGE,
D.W. (1978) Immunological induction of T lymphocytes:
Role of antigen and the lymphocyte costimulator. Blood
Cells 4 : 395-404.
- LAFFERTY, K.J., ANDRUS, L. and PROWSE, S.J. (1980) Role of
lymphokine and antigen in the control of specific T cell
responses. Immunological Rev. 51 : 279-314.
- LANE, J.T.L., JACKSON, L. and LING, N.R. (1975) Lymphocyte
stimulation with skin cells. A function of maturation.
Transplantation 19 : 250-259.
- LECHLER, R.I. and BATCHELOR, J.R. (1982) Restoration of
immunogenicity to passenger cell-depleted kidney allo-
grafts by the addition of donor strain dendritic cells.
J. Exp. Med. 155 : 31-41.
- LEDBETTER, J.A., ROUSE, R.V., MICKLEM, H.S. and HERZENBERG,
L.A. (1980) T cell subsets defined by expression of
Lyt-1,2,3 and Thy-1 antigens. Two-parameter immuno-
fluorescence and cytotoxicity analysis with monoclonal
antibodies modifies current views. J. Exp. Med. 152 :
280-295.
- LEONARD, R.J., LAZAROW, A. and HEGRE, O.D. (1973)
Pancreatic islet transplantation in the rat. Diabetes
22 : 413-428.
- LEUNG, K.-N., ADA, G.L. and MCKENZIE, I.F.C. (1980)
Specificity, Ly phenotype, and H-2 compatibility
requirements of effector cells in delayed-type hyper-
sensitivity responses to murine influenza virus
infection. J. Exp. Med. 151 : 815-826.

- LIEW, F.Y. and SIMPSON, E. (1980) Delayed-type hypersensitivity responses to H-Y: Characterization and mapping of *Ir* genes. *Immunogenetics* 11 : 255-266.
- LINDAHL-KIESSLING, K. and SÄFWENBERG, J. (1971) Inability of UV-irradiated lymphocytes to stimulate allogeneic cells in mixed lymphocyte culture. *Int. Arch. Allergy* 41 : 670-678.
- LINDER, O.E.A. (1961) Comparisons between survival of grafted skin, ovaries, and tumors in mice across histocompatibility barriers of different strength. *J. Nat. Cancer Inst.* 27 : 351-373.
- LITTLE, C.C. (1921-1922) The relation of genetics to the problems of cancer research. *Harvey Lect.* 17 : 65-88.
- LITTLE, C.C. (1924) The genetics of tissue transplantation in mammals. *J. Cancer Res.* 8 : 75-95.
- LOEB, L. (1918a) The grafting of tissues into nearly related individuals in the rat, and the mode of inheritance of individuality differentials. *J. Med. Res.* 38 : 393-420.
- LOEB, L. (1918b) Multiple transplantations of the thyroid and the lymphocytic reaction. *J. Med. Res.* 39 : 71-91.
- LOEB, L. (1930) Transplantation and individuality. *Physiol. Rev.* 10 : 547-616.
- LOVELAND, B.E. and MCKENZIE, I.F.C. (1982a) Which T cells cause graft rejection? *Transplantation* 33 : 217-221.
- LOVELAND, B.E. and MCKENZIE, I.F.C. (1982b) Cells mediating graft rejection in the mouse. III. Ly-1⁺ precursor T cells generate skin graft rejection. *Transplantation* 33 : 407-410.
- LOVELAND, B.E. and MCKENZIE, I.F.C. (1982c) Cells mediating graft rejection in the mouse. II. The Ly phenotypes of cells producing tumor allograft rejection. *Transplantation* 33 : 174-180.

- LOVELAND, B.E., HOGARTH, P.M., CEREDIG, Rh. and McKENZIE, I.F.C. (1981) Cells mediating graft rejection in the mouse. I. Lyt-1 cells mediate skin graft rejection. *J. Exp. Med.* 153 : 1044-1057.
- LUEKER, D.C. and SHARPTON, T.R. (1974) Survival of ovarian allografts following maintenance in organ culture. *Transplantation* 18 : 457-458.
- McKENZIE, I.F.C. and SNELL, G.D. (1973) Comparative immunogenicity and enhanceability of individual H-2K and H-2D specificities of the murine histocompatibility-2 complex. *J. Exp. Med.* 138 : 259-277.
- McKENZIE, I.F.C. and HENNING, M.M. (1978) The differential destructive and enhancing effects of anti-H-2K, H-2D and anti-Ia antisera on murine skin allografts. *J. Exp. Med.* 147 : 611-616.
- McEVOY, R.C. (1981) Fetal rat pancreas in organ culture: Effect of exogenous insulin on the development of the islet cells. *Horm. Metab. Res.* 13 : 5-8.
- McEVOY, R.C. and HEGRE, O.D. (1978) Syngeneic transplantation of fetal rat pancreas. II. Effect of insulin treatment on the growth and differentiation of pancreatic implants fifteen days after transplantation. *Diabetes* 27 : 988-995.
- McEVOY, R.C. and HEGRE, O.D. (1979) Syngeneic transplantation of fetal rat pancreas. III. Effect of insulin treatment on the growth and differentiation of the pancreatic implants after reversal of diabetes. *Diabetes* 28 : 141-146.
- McEVOY, R.C., SCHMITT, R.V. and HEGRE, O.D. (1978) Syngeneic transplantation of fetal rat pancreas. I. Effect of insulin treatment on the reversal of alloxan diabetes. *Diabetes* 27 : 982-987.
- MAHMOUD, A.A.F., RODMAN, H.M., MANDEL, M.A. and WARREN, K.S. (1976) Induced and spontaneous diabetes mellitus and suppression of cell-mediated immunologic responses. Granuloma formation, delayed dermal reactivity, and allograft rejection. *J. Clin. Invest.* 57 : 362-367.

- MAIN, R.K. and COLE, L.J. (1966) Immunological interaction between allogeneic and xenogeneic rodent spleen cells in culture: Stimulation of DNA synthesis. Clin. Exp. Immunol. 1 : 415-424.
- MANDEL, T.E. and HIGGINBOTHAM, L. (1979) Organ culture and transplantation of fetal mouse pancreatic islets. Transplant. Proc. 11 : 1505-1506.
- MANDEL, T.E., COLLIER, S., CARTER, W., HIGGINBOTHAM, L. and MARTIN, F.I.R. (1980) Effect of *in vitro* glucose concentration on fetal mouse pancreas cultures used as grafts in syngeneic diabetic mice. Transplantation 30 : 231-233.
- MANDEL, T.E., HOFFMAN, L. and CARTER, W.M. (1981) Long-term isografts of cultured fetal mouse pancreatic islets. The oncogenic effects of Streptozotocin and the prevention of diabetic renal complications. Am. J. Pathol. 104 : 227-236.
- MANDEL, T.E., HOFFMAN, L., COLLIER, S., CARTER, W.M. and KOULMANDA, M. (1982) Organ culture of fetal mouse and fetal human pancreatic islets for allografting. Diabetes 31 (Suppl. 4) : 39-47.
- MANNING, D.D., REED, N.D. and SHAFFER, C.F. (1973) Maintenance of skin xenografts of widely divergent phylogenetic origin on congenitally athymic (nude) mice. J. Exp. Med. 138 : 488-494.
- MARQUET, R.L. and HEYSTEK, G.A. (1981) Induction of suppressor cells by donor-specific blood transfusions and heart transplantation in rats. Transplantation 31 : 272-274.
- MATAS, A.J., SUTHERLAND, D.E.R., STEFFES, M.W. and NAJARIAN, J.S. (1976) Islet transplantation using neonatal rat pancreata: Quantitative studies. J. Surg. Res. 20 : 143-147.
- MEDAWAR, P.B. (1944) The behaviour and fate of skin autografts and skin homografts in rabbits. J. Anat. 78 : 176-199.

- MEDAWAR, P.B. (1946) Immunity to homologous grafted skin. II. The relationship between the antigens of blood and skin. *Brit. J. Exp. Pathol.* 27 : 15-24.
- MEDAWAR, P.B. (1956-1957) The immunology of transplantation. *Harvey Lect.* 52 : 144-176.
- MEDAWAR, P.B. (1957) The uniqueness of the individual (Chapter 8). In: *The uniqueness of the individual*. Methuen, London, pp. 143-185.
- MEDAWAR, P.B. (1963) Introduction: Definition of the immunologically competent cell. In: *The immunologically competent cell : Its nature and origin*. Ciba Foundation Study Group No. 16. Wolstenholme, G.E.W. and Knight, J. (Eds). Churchill, London, pp. 1-3.
- MILGROM, F. (1977) Role of humoral antibodies in transplantation. *Transplant. Proc.* 9 : 721-727.
- MILLARD, P.R., GARVEY, J.F.W., JEFFERY, E.L. and MORRIS, P.J. (1980) The grafted fetal rat pancreas. Features of development and rejection. *Am. J. Pathol.* 100 : 209-224.
- MILLER, J.F.A.P. (1961) Immunological function of the thymus. *Lancet* 2 : 748-749.
- MILLER, J.F.A.P. (1962) Role of the thymus in transplantation tolerance and immunity. In: *Transplantation*. Ciba Foundation Symposium. Wolstenholme, G.E.W. and Cameron, M.P. (Eds). Churchill, London, pp. 384-403.
- MILLER, J.F.A.P. and OSOBA, D. (1967) Current concepts of the immunological function of the thymus. *Physiol. Rev.* 47 : 437-520.
- MILLER, J.F.A.P., VADAS, M.A., WHITELOW, A. and GAMBLE, J. (1976) Role of major histocompatibility complex gene products in delayed-type hypersensitivity. *Proc. Natl. Acad. Sci. U.S.A.* 73 : 2486-2490.

- MITCHISON, N.A. (1953) Passive transfer of transplantation immunity. *Nature* 171 : 267-268.
- MITCHISON, N.A. (1954) Passive transfer of transplantation immunity. *Proc. Roy. Soc. B* 142 : 72-87.
- MITCHISON, N.A. (1959) Blood transfusion in fowl: An example of immunological tolerance requiring the persistence of antigen. In: *Biological problems of grafting*. Albert, F. and Medawar, P.B. (Eds). Blackwell, Oxford, pp. 239-259.
- MOORE, G.E., GERNER, R.E. and FRANKLIN, H.A. (1967) Culture of normal human leukocytes. *J. Am. Med. Assoc.* 199 : 519-524.
- MORRIS, P.J., FINCH, D.R., GARVEY, J.F., POOLE, M.D. and MILLARD, P.R. (1980) Suppression of rejection of allogeneic islet tissue in the rat. *Diabetes* 29 (Suppl. 1): 107-112.
- MULLEN, Y. (1980) Specific immunosuppression for fetal pancreas allografts in rats. *Diabetes* 29 (Suppl. 1) : 113-120.
- MULLEN, Y. and SHINTAKU, I.P. (1980) Fetal pancreas allografts for reversal of diabetes in rats. I. Allograft survival in nonimmunosuppressed recipients. *Transplantation* 29 : 35-42.
- MURRELL, L.R. (1966) Mammalian pancreatic islet tissue in organ culture. I. Methods of culture and *in vitro* histogenesis. *Exp. Cell Res.* 41 : 350-364.
- NAJARIAN, J.S., SUTHERLAND, D.E.R. and STEFFES, M.W. (1975) Isolation of human islets of Langerhans for transplantation. *Transplant. Proc.* 7 : 611-613.
- NAJI, A. and BARKER, C.F. (1976) The influence of histocompatibility and transplant site on parathyroid allograft survival. *J. Surg. Res.* 20 : 261-267.
- NAJI, A., SILVERS, W.K. and BARKER, C.F. (1979) Effect of culture in 95% O₂ on the survival of parathyroid allografts. *Surg. Forum* 30 : 109-111.

- NAJI, A., FRANGIPANE, L., BARKER, C.F. and SILVERS, W.K.
(1981a) Survival of H-Y-incompatible endocrine grafts in mice and rats. Transplantation 31 : 145-147.
- NAJI, A., SILVERS, W.K. and BARKER, C.F. (1981b) Influence of organ culture on the survival of major histocompatibility complex-compatible and incompatible parathyroid allografts in rats. Transplantation 32 : 296-298.
- NASH, J.R. and BELL, P.R.F. (1979) Effect of macrophage suppression on the survival of islet allografts. Transplant.Proc. 11 : 986-988.
- NICHOLS, W.K., SPELLMAN, J.B., VANN, L.L. and DAYNES, R.A. (1979) Immune responses of diabetic animals. Direct immunosuppressant effects of Streptozotocin in mice. Diabetologia 16 : 51-57.
- NINNEMANN, J.L. and GOOD, R.A. (1974) Allogeneic transplantation of organ cultures without immunosuppression. Transplantation 18 : 1-5.
- OLUWOLE, S., HARDY, M.A., WANG, T., SATAKE, K., TODD, G., REEMTSMA, K. and NOWYGRAD, R. (1980) Donor pretreatment: Rat heart allograft survival and measurement of passenger leukocyte depletion with Indium-111. Transplantation 30 : 31-33.
- PARISH, C.R., CHILCOTT, A.B. and MCKENZIE, I.F.C. (1976) Low molecular weight *Ia* antigens in normal mouse serum. II. Demonstration of their T cell origin. Immunogenetics 3 : 129-137.
- PARR, E.L., LAFFERTY, K.J., BOWEN, K.M. and MCKENZIE, I.F.C. (1980a) H-2 complex and *Ia* antigens on cells dissociated from mouse thyroid glands and islets of Langerhans. Transplantation 30 : 142-148.
- PARR, E.L., BOWEN, K.M. and LAFFERTY, K.J. (1980b) Cellular changes in cultured mouse thyroid glands and islets of Langerhans. Transplantation 30 : 135-141.
- PARR, E.L., OLIVER, J.R. and KING, N.J.C. (1982) The surface concentration of H-2 antigens on mouse pancreatic β -cells and islet cell transplantation. Diabetes 31 (Suppl. 4) : 1-10.

- PREHN, R.T. and MAIN, J.M. (1954) A comparison between heterozygous and homozygous skin homografts. *J. Nat. Cancer Inst.* 15 : 191-194.
- PROWSE, S.J., WARREN, H.S., AGOSTINO, M. and LAFFERTY, K.J. (1982) Transfer of sensitised Lyt 2⁺ cells triggers acute rejection of pancreatic islet allografts. *Aust. J. Exp. Biol. Med. Sci.* In press.
- RAAF, J.H., VAN PILSUM, J.F. and GOOD, R.A. (1976) Fresh and cultured thyroid gland: Survival and function after implantation. *Ann. Surg.* 183 : 146-156.
- RABINOVITCH, A., ALEJANDRO, R., NOEL, J., BRUNSCHWIG, J.-P. and RYAN, U.S. (1982) Tissue culture reduces *Ia* antigen-bearing cells in rat islets and prolongs islet allograft survival. *Diabetes* 31 (Suppl. 4) : 48-54.
- RAKIETEN, N., RAKIETEN, M.L. and NADKARNI, M.V. (1963) Studies on the diabetogenic action of Streptozotocin. *Cancer Chemother.* 29 : 91-98.
- RECKARD, C.R. and BARKER, C.F. (1973) Transplantation of isolated pancreatic islets across strong and weak histocompatibility barriers. *Transplant. Proc.* 5 : 761-763.
- ROBERTS, P.J. and HÄYRY, P. (1977) Effector mechanisms in allograft rejection. II. Density, electrophoresis, and size fractionation of allograft-infiltrating cells demonstrating several classes of killer cells. *Cell. Immunol.* 30 : 236-253.
- ROSENSTREICH, D.L. and WAHL, S.M. (1979) Cellular sources of lymphokines. In: *Biology of the lymphokines*. Cohen, S., Pick, E. and Oppenheim, J.J. (Eds). Academic Press, New York, pp. 209-242.
- RUSSELL, P.S. and GITTES, R.F. (1959) Parathyroid transplants in rats. A comparison of their survival time with that of skin grafts. *J. Exp. Med.* 109 : 571-588.

- SCHEID, M., BOYSE, E.A., CARSWELL, E.A. and OLD, L.J. (1972)
Serologically demonstrable alloantigens of mouse
epidermal cells. *J. Exp. Med.* 135 : 938-955.
- SCHELLEKENS, P.T.A. and EIJSVOOGEL, V.P. (1970) Lymphocyte
transformation *in vitro*. III. Mechanism of stimulation
in the mixed lymphocyte culture. *Clin. Exp. Immunol.* 7 :
229-239.
- SCHWEISTHAL, M.R., WELLS, L.J. and CÉAS, M.P. (1965)
Development of islets and acini from the explanted
primordium of the pancreas of the rat embryo.
Anat. Rec. 151 : 93-106.
- SCOLLAY, R.G., LAFFERTY, K.J. and POSKITT, D.C. (1974)
Allogeneic stimulation modulates the strength of
transplantation antigen. *Transplantation* 18 : 6-13.
- SCOTHORNE, R.J. (1957) Studies on the response of the
regional lymph node to skin homografts. *Ann. N.Y. Acad.*
Sci. 64 : 1028-1039.
- SILVERS, W.K. (1968) Studies on the induction of tolerance
of the H-Y antigen in mice with neonatal skin grafts.
J. Exp. Med. 128 : 69-83.
- SILVERS, W.K., FLEMING, H.L., NAJI, A. and BARKER, C.F. (1982)
Evidence for major histocompatibility complex restriction
in transplantation immunity. *Proc. Natl. Acad. Sci.*
U.S.A. 79 : 171-174.
- SIMONSEN, M. (1962) Graft versus host reactions. Their
natural history, and applicability as tools of research.
Progr. Allergy 6 : 349-467.
- SMITH, R.T. and BRIDGES, R.A. (1958) Immunological
unresponsiveness in rabbits produced by neonatal
injection of defined antigens. *J. Exp. Med.* 108 :
227-250.
- SNELL, G.D. (1948) Methods for the study of histocompatibility
genes. *J. Genetics* 49 : 87-103.

- SNELL, G.D. (1952) Enhancement and inhibition of the growth of tumor homoiotransplants by pretreatment of the hosts with various preparations of normal and tumor tissue. J. Nat. Cancer Inst. 13 : 719-729.
- SNELL, G.D. (1957a) Incompatibility reactions to tumor homotransplants with particular reference to the role of the tumor. Cancer Res. 17 : 2-10.
- SNELL, G.D. (1957b) The homograft reaction. Ann. Rev. Microbiol. 11 : 439-458.
- SNELL, G.D. (1971) The histocompatibility systems. Transplant. Proc. 3 : 1133-1138.
- SOLLINGER, H.W. and BACH, F.H. (1976) Collaboration between *in vivo* responses to LD and SD antigens of major histocompatibility complex. Nature 259 : 487-488.
- SOLLINGER, H.W., BURKHOLDER, P.M., RASMUS, W.R. and BACH, F.H. (1977) Prolonged survival of xenografts after organ culture. Surgery 81 : 74-79.
- SPENCE, R.K., PERLOFF, L.J. and BARKER, C.F. (1977) The role of the implant site in rat fetal pancreas transplantation. Trans. Am. Soc. Artif. Intern. Organs 23 : 352-357.
- SPENCE, R.K., PERLOFF, L.J. and BARKER, C.F. (1979) Fetal pancreas in treatment of experimental diabetes in rats. Transplant. Proc. 11 : 533-536.
- SPRENT, J. and MILLER, J.F.A.P. (1972a) Interaction of thymus lymphocytes with histoincompatible cells. III. Immunological characteristics of recirculating lymphocytes from activated thymus cells. Cell. Immunol. 3 : 213-230.
- SPRENT, J. and MILLER, J.F.A.P. (1972b) Interaction of thymus lymphocytes with histoincompatible cells. II. Recirculating lymphocytes derived from antigen-activated thymus cells. Cell. Immunol. 3 : 385-404.
- SPRENT, J. and KORNGOLD, R. (1981) Immunogenetics of graft-versus-host reactions to minor histocompatibility antigens. Immunology Today 2 : 189-195.

- STEINMAN, R.M. and WITMER, M. (1978) Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc. Natl. Acad. Sci.* 75 : 5132-5136.
- STEINMULLER, D. (1967) Immunisation with skin isografts taken from tolerant mice. *Science* 158 : 127-129.
- STEINMULLER, D. (1969) Allograft immunity produced with skin isografts from immunologically tolerant mice. *Transplant. Proc.* 1 : 593-596.
- STEINMULLER, D. (1980) Passenger leukocytes and the immunogenicity of skin allografts. *J. Invest. Dermatol.* 75 : 107-115.
- STEINMULLER, D. (1981) Passenger leukocytes and the immunogenicity of skin allografts: A critical reevaluation. *Transplant. Proc.* 13 : 1094-1098.
- STEINMULLER, D. and HART, E.A. (1971) Passenger leukocytes and induction of allograft immunity. *Transplant. Proc.* 3 : 673-675.
- STEINMULLER, D., TYLER, J.D. and DAVID, C.S. (1981) Cell-mediated cytotoxicity to non-MHC alloantigens on mouse epidermal cells. II. Genetic basis of the response of C3H mice. *J. Immunol.* 126 : 1754-1758.
- STEINMULLER, D., TYLER, J.D., WADDICK, K.G. and BURLINGHAM, W.J. (1982) Epidermal alloantigen and the survival of mouse skin allografts. *Transplantation* 33 : 308-313.
- STINGL, G., KATZ, S.I., CLEMENT, L., GREEN, I. and SCHEVACH, E.M. (1978) Immunologic functions of *Ia*-bearing epidermal Langerhans cells. *J. Immunol.* 121 : 2005-2013.
- STONE, H.B., OWINGS, J.C. and GEY, G.O. (1934) Transplantation of living grafts of thyroid and parathyroid glands. *Ann. Surgery* 100 : 613-628.
- STREILEIN, J.W. and BERGSTRESSER, P.R. (1980) *Ia* antigens and epidermal Langerhans cells. *Transplantation* 30 : 319-323.

- STROBER, S. and GOWANS, J.L. (1965) The role of lymphocytes in the sensitization of rats to renal homografts. *J. Exp. Med.* 122 : 347-360.
- STROM, T.B., TILNEY, N.L., CARPENTER, C.B. and BUSCH, G.J. (1975) Identity and cytotoxic capacity of cells infiltrating renal allografts. *N. Engl. J. Med.* 292 : 1257-1263.
- STROM, T.B., TILNEY, N.L., PARADYSZ, J.M., BANCEWICZ, J. and CARPENTER, C.B. (1977) Cellular components of allograft rejection: Identity, specificity, and cytotoxic function of cells infiltrating acutely rejecting allografts. *J. Immunol.* 118 : 2020-2026.
- STUART, F.P., BASTIEN, E., HOLTER, A., FITCH, F.W. and ELKINS, W.L. (1971) Role of passenger leukocytes in the rejection of renal allografts. *Transplant. Proc.* 3 : 461-464.
- SUMMERLIN, W.T., BROUTBAR, C., FOANES, R.B., PAYNE, R., STUTMAN, O., HAYFLICK, L. and GOOD, R.A. (1973) Acceptance of phenotypically differing cultured skin in man and mice. *Transplant. Proc.* 5 : 707-710.
- SUTHERLAND, D.E.R. (1981a) Pancreas and islet transplantation. I. Experimental studies. *Diabetologia* 20 : 161-185.
- SUTHERLAND, D.E.R. (1981b) Pancreas and islet transplantation. II. Clinical trials. *Diabetologia* 20 : 435-450.
- SWINSCOW, T.D.V. (1980a) Statements of probability (Chapter 4). In: *Statistics at square one*. Brit. Med. Assoc., London, pp. 21-23.
- SWINSCOW, T.D.V. (1980b) Exact probability test (Chapter 9). In: *Statistics at square one*. Brit. Med. Assoc., London, pp. 54-57.
- TALMAGE, D.W. (1980) Effects of oxygen, temperature, and time of culture on the survival of mouse thyroid and pancreas allografts. *Diabetes* 29 (Suppl. 1) : 105-106.
- TALMAGE, D.W. and DART, G.A. (1978) Effect of oxygen pressure during culture on survival of mouse thyroid allografts. *Science* 200 : 1066-1067.

- TALMAGE, D.W., DART, G., RADOVICH, J. and LAFFERTY, K.J. (1976) Activation of transplant immunity: Effect of donor leukocytes on thyroid allograft rejection. *Science* 191 : 385-388.
- TALMAGE, D.W., WOOLNOUGH, J.A., HEMMINGSEN, H., LOPEZ, L. and LAFFERTY, K.J. (1977) Activation of cytotoxic T cells by nonstimulating tumor cells and spleen cell factor(s). *Proc. Natl. Acad. Sci. U.S.A.* 74 : 4610-4614.
- THOMSON, N.M., HANCOCK, W.W., LAFFERTY, K.J., KRAFT, N. and ATKINS, R.C. (1982) Organ culture reduces *Ia*-positive cells present within the human foetal pancreas. *Transplant. Proc.* In press.
- TYZZER, E.E. (1909) A study of inheritance in mice with reference to their susceptibility to transplantable tumors. *Am. J. Pathol.* 21 : 519-573.
- TYZZER, E.E. (1916) Tumor immunity. *J. Cancer Res.* 1 : 125-155.
- USADEL, K.H., SCHWEDES, U., BASTERT, G., STEINAU, U., KLEMPA, I., FASSBINDER, W. and SCHÖFFLING, K. (1980) Transplantation of human fetal pancreas. Experience in thymusaplastic mice and rats and in a diabetic patient. *Diabetes* 29 (Suppl. 1) : 74-79.
- VALENTE, U., FERRO, M., BAROCCI, S., CAMPISI, C., PARODI, F., CATALDI, L., ARCURI, V. and TOSATTI, E. (1980) Report of clinical cases of human fetal pancreas transplantation. *Transplant. Proc.* 12 : 213-217.
- VON WILLEBRAND, E. and HÄYRY, P. (1978) Composition and *in vitro* cytotoxicity of cellular infiltrates in rejecting human kidney allografts. *Cell. Immunol.* 41 : 358-372.
- WACHTEL, S.S. and SILVERS, W.K. (1971) Skin homografts: Tolerogenic versus immunogenic influences in mice. *J. Exp. Med.* 133 : 921-937.
- WAGNER, H. and BOYLE, W. (1972) Subcellular mouse allo-antigens: Cytotoxic immune responses and specific blocking *in vitro*. *Nature (New Biol.)* 240 : 92-94.

- WAGNER, H., HARRIS, A.W. and FELDMANN, M. (1972) Cell-mediated immune response *in vitro*. II. The role of thymus and thymus-derived lymphocytes. *Cell. Immunol.* 4 : 39-50.
- WARREN, R.P., LOFGREEN, J.S. and STEINMULLER, D. (1973) Factors responsible for the differential survival of heart and skin allografts in inbred rats. *Transplantation* 16 : 458-465.
- WELLS, L.J., SCHWEISTHAL, M.R., MARX, R.N., MCKAY, M., SACCOMAN, F.M. and LAZAROW, A. (1967) Effects of different levels of glucose upon the development of granulated beta cells in cultures of pancreatic primordia from normal rat embryos. *Diabetes* 16 : 839-845.
- WELSH, K.I., BATCHELOR, J.R., MAYNARD, A. and BURGOS, H. (1979) Failure of long surviving, passively enhanced kidney allografts to provoke T-dependent alloimmunity. II. Retransplantation of (AS x AUG) F₁ kidneys from AS primary recipients into (AS x WF) F₁ secondary hosts. *J. Exp. Med.* 150 : 465-470.
- WETTSTEIN, P.J. and FRELINGER, J.A. (1977) H-2 effects on cell-cell interactions in the response to single non-H-2 alloantigens. II. H-2D region control of H-7.1-specific stimulator function in mixed lymphocyte culture and susceptibility to lysis by H-7.1-specific cytotoxic cells. *J. Exp. Med.* 146 : 1356-1366.
- WETTSTEIN, P.J. and FRELINGER, J.A. (1980) H-2 effects on cell-cell interactions in the response to single non-H-2 alloantigens. *Immunogenetics* 10 : 211-225.
- WETTSTEIN, P.J., HAUGHTON, G. and FRELINGER, J.A. (1977) H-2 effects on cell-cell interactions in the response to single non-H-2 alloantigens. I. Donor H-2D region control of H-7.1-immunogenicity and lack of restriction *in vivo*. *J. Exp. Med.* 146 : 1346-1355.

- WHITE, E. and HILDEMAN, W.H. (1968) Allografts in genetically defined rats: Difference in survival between kidney and skin. *Science* 162 : 1293-1295.
- WIKTOROWICZ, K., ROBERTS, P.J. and HÄYRY, P. (1978) Effector mechanisms in allograft rejection. IV. In contrast to late cytotoxic cells, the early killer cells infiltrating mouse sponge matrix allografts are predominantly T lymphocytes. *Cell. Immunol.* 38 : 255-263.
- WILLIAMS, G.M., HUME, D.M., HUDSON, R.P., MORRIS, P.J., KANO, K. and MILGROM, F. (1968) "Hyperacute" renal-homograft rejection in man. *N. Engl. J. Med.* 279 : 611-618.
- WILSON, D.B. and NOWELL, P.C. (1970) Quantitative studies on the mixed lymphocyte interaction in rats. IV. Immunologic potentiality of the responding cells. *J. Exp. Med.* 131 : 391-407.
- WINN, H.J., BALDAMUS, C.A., JOOSTE, S.V. and RUSSELL, P.S. (1973) Acute destruction by humoral antibody of rat skin grafted to mice. The role of complement and polymorphonuclear leukocytes. *J. Exp. Med.* 137 : 893-910.
- WOODRUFF, M.F.A. and ANDERSON, N.F. (1964) The effect of lymphocyte depletion by thoracic duct fistula and administration of antilymphocyte serum on the survival of skin homografts in rats. *Ann. N.Y. Acad. Sci.* 120 : 119-128.
- WOOLNOUGH, J.A., MISKO, I.S. and LAFFERTY, K.J. (1979) Cytotoxic and proliferative lymphocyte responses to allogeneic and xenogeneic antigens *in vitro*. *Aust. J. Exp. Biol. Med. Sci.* 57 : 467-477.
- WORTIS, H.H. (1971) Immunological responses of 'nude' mice. *Clin. Expl. Immunol.* 8 : 305-317.
- WORTIS, H.H., NEHLSSEN, S. and OWEN, J.J. (1971) Abnormal development of the thymus in "nude" mice. *J. Exp. Med.* 134 : 681-692.

- YASUNAMI, Y., TAKAKI, R., FUKUMA, M., OKEDA, T. and KONOMI, K.
(1981) Adult human pancreatic endocrine cells in culture.
Insulin and glucagon release and influence of cold
storage. J. Lab. Clin. Med. 98 : 173-180.
- ZIEGLER, M.M., RECKARD, C.R., NAJI, A. and BARKER, C.F. (1975)
Extended function of isolated pancreatic islet isografts
and allografts. Transplant. Proc. 7 (Suppl. 1) : 743-745.
- ZITRON, I.M., ONO, J., LACY, P.E. and DAVIE, J.M. (1981a)
Active suppression in the maintenance of pancreatic islet
allografts. Transplantation 32 : 156-158.
- ZITRON, I.M., ONO, J., LACY, P.E. and DAVIE, J.M. (1981b)
The cellular stimuli for the rejection of established
islet allografts. Diabetes 30 : 242-246.